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BET INHIBITION AS A STRATEGY FOR ENHANCING THE RESPONSE TO ESTROGEN ANTAGONISM/CDK4-6 INHIBITION IN ER POSITIVE BREAST CANCER

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Abbreviations

AV	Annexin V
AMBRA 1	Activating Molecule in Beclin1-Regulated Autophagy
Baf	Bafilomycin A1
Bak	Bcl-2 Homologous Killer
Bax	Bcl-2-Associated X Protein
BCL-2	B-Cell Lymphoma 2
BCL-W	Bcl-2-Like Protein 2
BCL-XL	B-Cell Lymphoma-Extra Large
BET	Bromo- and Extra-Terminal Domain

BNIP3	Bcl-2 Interacting Protein 3
BRD 2/3/4	Bromodomain-Containing Protein 2/3/4
C12FDG	5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside
CDK	Cyclin-dependent Kinase
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ER+	Estrogen Receptor positive
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
IL-1β	Interleukin-1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
KD	Knock Down
KO	Knockout
LC3 I/II	Light Chain Microtubule-Associated Protein
MCL-1	Myeloid Cell Leukemia-1
MMP	Matrix Metalloprotease
mTOR	Mammalian Target of Rapamycin
PBS	Phosphate Buffered Solution
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase

pRB	Retinoblastoma protein
PROTAC	Proteolysis Targeting Chimera
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SA-β-Gal	Senescence-Associated-β-Galactosidase
SAHF	Senescence-Associated Heterochromatic Foci
SASP	Senescence-Associated Secretory Phenotype
SD	Standard Deviation
SEM	Standard Error of the Mean
siRNA	Small Interfering RNA
shRNA	Short Hairpin RNA
UVRAG	UV Radiation Resistance-Associated Gene Protein
WT	Wild type

Abstract

Breast cancer is the most commonly occurring malignancy in females, accounting for the second most common cause of cancer-related deaths. ER+ breast cancer constitutes approximately 70% of all breast cancer cases. The standard care of therapy comprises endocrine antagonists together with adjuvant therapy; however, the recovery from the therapy-suppressive state is associated with further drug resistance together with increasing tumor aggressiveness and with possible metastasis. Autophagy induction has been shown to be associated with various therapeutic modalities, with different functions, including cytotoxic, cytoprotective, cytostatic as well as non-protective forms. The triggering of autophagy may be responsible for resistance development and apoptosis suppression in the form known as cytoprotective autophagy, whereupon autophagy inhibition would represent a possible strategy to increase the effectiveness of various chemotherapeutic agents. Initially, we showed that Fulvestrant plus Palbociclib, one standard care of therapy, induces autophagy; therefore, we hypothesized that autophagy inhibition could increase the effectiveness of this combination. However, autophagy inhibition either genetically our pharmacologically resulted in only slight sensitization, suggesting that the autophagy role here is largely non-protective. Alternatively, we showed that Fulvestrant plus Palbociclib drives the ER+ cells into senescence, a state of reversible growth arrest, from which the cells begin to recover between days 12-18. Another strategy that being investigated to increase the effectiveness of endocrine therapies is by utilizing senescence targeting drugs including senomorphics, senostatics as well as senolytics. Furthermore, an association between senescence and epigenetic targets is demonstrable in that a member of the BET family, BRD4, has been shown to be overexpressed in breast cancer together with its downstream effector c-Myc, making it a possible therapeutic target. Therefore, we hypothesized that the BRD4

inhibitors, ARV-825 or ABBV-744, could increase the effectiveness of endocrine therapies, Tamoxifen and Fulvestrant plus Palbociclib. ARV-825 showed promising results together with Tamoxifen and Fulvestrant plus Palbociclib, with a potential senolytic activity against the senescent population induced by Fulvestrant plus Palbociclib. Moreover, ABBV-744 showed a p53-dependent growth inhibition when combined with Fulvestrant plus Palbociclib; however, ABBV-744 did not affect the proliferation or the viability of Tamoxifen-treated cells. Thses studies highlight the effectiveness of ARV-825 *in vitro*, and the need for testing the drug in resistant cell lines as well as *in vivo* using mice models.

Disclosures

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f) Some parts of this work also appear in Ryan Finnegan's thesis. Experimental data that was generated by Ryan Finnegan alone is indicated by the symbol (#). Data that was generated by Ahmed M. Elshazly together with Ryan Finnegan is not specifically indicated. Data that was generated in Dr. Knudsen's laboratory as well as in Dr. Schoenlein's laboratory are indicated.

Chapter 1: General Introduction

1.1.Breast Cancer

1.1.1. Breast Cancer Overview

Breast cancer (BC) is considered the most frequently diagnosed cancer in females and the second most common cause of cancer-related deaths, accounting for 30% of female tumors alone [1]. In the U.S, it is estimated that approximately 40,000 women die from breast cancer each year [2]. Moreover, in 2020, 2.3 million women were diagnosed with BC worldwide, Near the end of 2020, 7.8 million women diagnosed with BC in the previous five years were alive, making it the most prevalent cancer globally [3].

Breast cancer prognosis and classification rely not only on tumor morphology but also on the expression levels of three proteins, specifically the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2). Tumors that do not express any of these proteins are classified as triple-negative breast cancers, a form of the disease that is particularly difficult to treat [4]. Estrogen receptors (ERs) are nuclear proteins regulating the expression of various genes, with approximately 80% of breast cancer cases being estrogen receptor (ER)-positive [5].

1.1.2. Treatment Options for ER+ Breast Cancer and Their Mechanisms of Action

The standard of care for estrogen receptor (ER)-positive breast cancer is endocrine therapy in combination with adjuvant therapy, which has reduced relapse and mortality by up to 40% [6]. The clinically available endocrine therapies include selective estrogen receptor

modulators (SERMS) such as Tamoxifen (TAM), selective estrogen receptor degraders (SERDs), such as Fulvestrant [7], and aromatase inhibitors (AIs) such as letrozole [8].

Tamoxifen (TAM) is one of the oldest and most frequently utilized (SERMS), which competes with estrogen at the receptor site, blocking the estrogen promotional role in breast cancer [9] (**Figure 1.1**). TAM is typically prescribed to treat pre-menopausal women with early-stages of hormone receptor-positive breast cancer after surgery to reduce disease recurrence. SERDs bind to the ER and either block estrogen from binding to the receptor or alter the shape of the ER such that ER function is compromised. Typically, SERD binding to the ER results in ER degradation (**Figure 1.1**). The most common SERD is Fulvestrant, which is used occasionally as a monotherapy in early hormone receptor positive breast cancer cases in post-menopausal women. It is also used in advanced stage breast cancer when other hormonal therapies fail [8].

A third class of hormonal therapy involves aromatase inhibitors, which block the enzyme aromatase (**Figure 1.1**). Aromatase converts androgens into estrogens via a mechanism referred to as aromatization. Blockade of aromatase is a therapy primarily used for the treatment of breast cancer in postmenopausal women that are producing small amounts of testosterone and testosterone precursors from the adrenal gland. The most commonly used aromatase inhibitors are the steroidal, Exemestane, and the non-steroidal, Letrozole, and Anastrozole [10].

Cell cycle checkpoints including cyclin-dependent kinases CDK4 and CDK6 are often deregulated in different tumors and are considered one of the key cancer hallmarks. Selective targeting of CDK4/6 is an effective strategy which has shown promising preclinical and clinical results in numerous solid tumors [11]. CDK 4/6 inhibitors such as Palbociclib hamper cell cycle

progression by interfering with CDK-cyclin complexes, blocking G1/S cell cycle transition [12, 13] (**Figure 1.1**) and are currently being used as adjuvant therapy with either SERDs or AIs.

Unfortunately, while treatment of early ER-positive breast cancer with SERMs, SERDs, and AIs can reduce recurrence for up to 5 years, resistance to hormone therapy is common, and most cases eventually result in metastatic disease progression [5], [6].

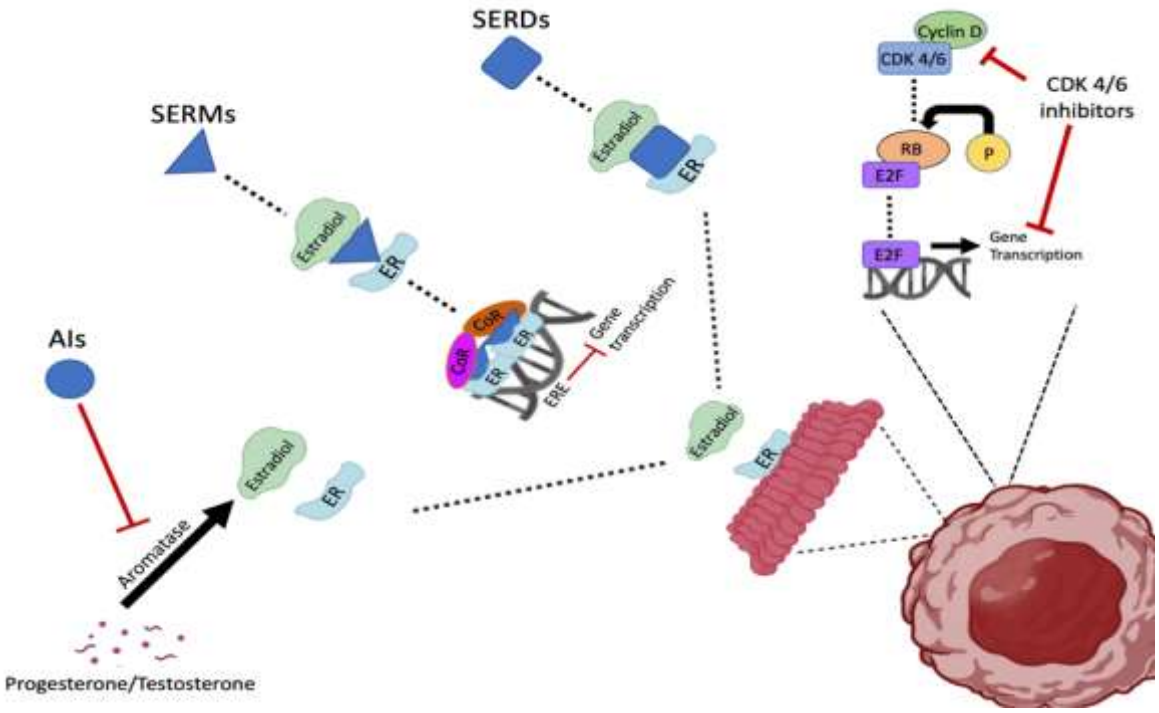


Figure 1.1 The effects of endocrine therapies (SERDs, SERMs, and AIs) and CDK 4/6 inhibitors on the ER pathway and gene transcription. SERDs block ER function by binding to the complex, resulting in degradation of the ER, SERMs prevent ER function by binding to ER to inactivate the complex, while aromatase inhibitors block ER function by inhibiting the synthesis of estradiol. CDK4/6 inhibitors prevent cell cycle progression by blocking the formation of CDK4/6 and cyclin D complex, which leads to the inhibition of gene transcription.

1.2. Apoptosis

One of the main goals in treating ER+ breast cancer is to induce cell death. The most common form of programmed cell death in the field of cancer biology is apoptosis. The term "apoptosis" is derived from the Greek words meaning "dropping off", which referred to the falling of leaves from trees in autumn. During apoptosis, the cells actively pursue different pathways toward death upon receiving specific signals [14]. Apoptotic cells exhibit various morphological alterations, affecting both nucleus and cytoplasm [15, 16] including, chromatin condensation, nuclear fragmentation, cell rounding, as well as decline in cellular volume (pyknosis) [17]. The plasma membrane remains intact throughout the total process; however, at the later stage of apoptosis, the cells acquire further morphological features, such as membrane blebbing, structural modification of cytoplasmic organelles and a loss of membrane integrity [17]. These alterations are usually followed by the engulfment of apoptotic cells by phagocytes before apoptotic bodies occur [18, 19].

Apoptosis occurs through two main pathways, intrinsic and extrinsic apoptosis. The intrinsic pathway is initiated within the cell upon receiving internal stimuli such as DNA damage, hypoxia, and severe oxidative stress [20]. This pathway is tightly regulated by Bcl-2 family proteins. There are two main groups of the Bcl-2 proteins, namely the pro-apoptotic proteins (e.g. Bax, Bak, and Bad) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-x_L, Bcl-W, and Mcl-1) [21]. While the anti-apoptotic proteins suppress apoptosis by blocking the mitochondrial release of cytochrome-c, the pro-apoptotic proteins act by promoting such release. Among apoptotic factors that are released from the mitochondrial inter-membrane space into the cytoplasm are apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), and direct IAP Binding protein with Low pI (DIABLO) [22]. Cytoplasmic release of

cytochrome c activates caspase 3 and the subsequent apoptosis induction via the formation of a complex known as the apoptosome which is made up of cytochrome c, Apaf-1 and caspase 9 [22]. On the other hand, Smac/DIABLO promotes caspase activation by binding to inhibitor of apoptosis proteins (IAPs) and suppressing their activity [22].

The extrinsic death receptor pathway begins with death ligand binds to a death receptor. Although several death receptors have been described, the best known death receptors is the type 1 TNF receptor (TNFR1) and a related protein called Fas (CD95) and their ligands, TNF and Fas ligand (FasL), respectively [23]. These death receptors have an intracellular death domain that recruits adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), as well as cysteine proteases like caspase 8 [24]. Binding of the death ligand to the death receptor results in the formation of a binding site for an adaptor protein and the whole ligand-receptor-adaptor protein complex is known as the death-inducing signaling complex (DISC) [25]. DISC then initiates the assembly and activation of pro-caspase 8. The activated form of the enzyme, caspase 8, is an initiator caspase, which initiates apoptosis by cleaving other downstream or executioner caspases [20].

1.3. Autophagy

1.3.1 Autophagy overview

Autophagy is a highly regulated catabolic process that plays a vital role in the maintenance of cellular homeostasis via the degradation of damaged organelles and cellular debris [26-28]. Autophagy occurs at a basal level in all cells and can be triggered by a variety of cellular signals and stresses including hypoxia, starvation, oxidative stress, endoplasmic reticulum (ER) stress and protein aggregation [29, 30]. In mammalian cells, there are three main classifications of autophagy: macroautophagy, microautophagy, and chaperone mediated autophagy (CMA). Each of these pathways, although morphologically distinct, ultimately involves the delivery of cargo to the lysosome. The lysosome provides the acidic environment and enzymes necessary for the degradation and recycling of cargo [31]. Macroautophagy (generally referred to as autophagy) is the autophagic pathway that is most frequently implicated in cancer cell resistance to therapy, including hormonal therapy resistance [32]. Macroautophagy is an evolutionarily conserved catabolic process through which cellular cargo is initially sequestered within a double membrane vesicle, prior to fusion with the lysosome. In this regard, macroautophagy is distinct from chaperone mediated autophagy and microautophagy in that each of these types of autophagy do not rely on an autophagosome to bring cargo to the lysosome [31]. To date, the components of autophagy and the required autophagic machinery, is encoded by 31 autophagy-related genes (ATG). Many of these genes and the autophagy pathway itself have been shown to be a necessary component of a number of cellular processes such as immune cell development, maintaining cell and tissue homeostasis, cellular metabolism, aging, and cancer [33]. Thus, it is not surprising that the impairment of autophagy in normal cells has been associated with multiple disease processes [34, 35].

1.3.2 Autophagy mechanism

The autophagy pathway is typically divided into separate stages: initiation of the double membrane phagophore, elongation and closure of the autophagosome membrane, fusion with the lysosome forming the autolysosome, and degradation of the intravesicular cargo. The initiation phase is regulated by the mammalian target of rapamycin, mTOR, which is a central component of two multiprotein complexes, designated mTORC1 and mTORC2. The mTORC1 complex is highly responsive to nutrient deprivation and limited amino acid availability, while mTORC2 [36-39] responds to growth factor availability. When mTORC1 activity is low, ULK1/2 (Unc-51-like kinase 1/2) is activated via dephosphorylation. The ULK1/2 complex is comprised of ULK1/2, FIP200, and ATG13. This complex, once assembled, phosphorylates members of class III PI3K complex, consisting of AMBRA1, Beclin1, VPS15/34, UVRAG, and ATG14 [40]. Phosphorylation of PI3KC, Beclin-1, and VPS34 is required for the initiation of phagophore nucleation, which is hypothesized to originate from multiple membrane sources, including the endoplasmic reticulum, mitochondria, Golgi apparatus, and recycling endosomes [36, 37, 41, 42].

Following the initial nucleation step, the phagophore is elongated by the ATG5/12 complex, which is conjugated by ATG16L and by the conjugation of active cytosolic LC3-I (encoded by ATG8) to phosphatidylethanolamine (PE), generating LC3-II. Conjugation with PE requires sequential activation of ATG7, ATG3, and the ATG5/12 complex [38]. Prior to LC3-I conjugation, cleavage of the C-terminal region of the inactive preform of LC3 is mediated by ATG4B protease. LC3-II is recruited to the phagophore membrane and is required for elongation of the inner and outer membranes of the autophagosome. Following phagophore maturation, the autophagosome fuses with the lysosome, resulting in the formation of an autolysosome, leading

to the degradation of the autophagic cargo, along with LC3-II. Thus, LC3-II turnover is often utilized as a marker for autophagosome formation and functional autophagic flux [39]. Another important player in the autophagic process is Sequestosome 1 (p62/SQSTM1), a ubiquitin and LC3 binding protein, which is also degraded during autolysosomal turnover, providing independent measure of functional autophagy [8] (**Figure 1.2**).

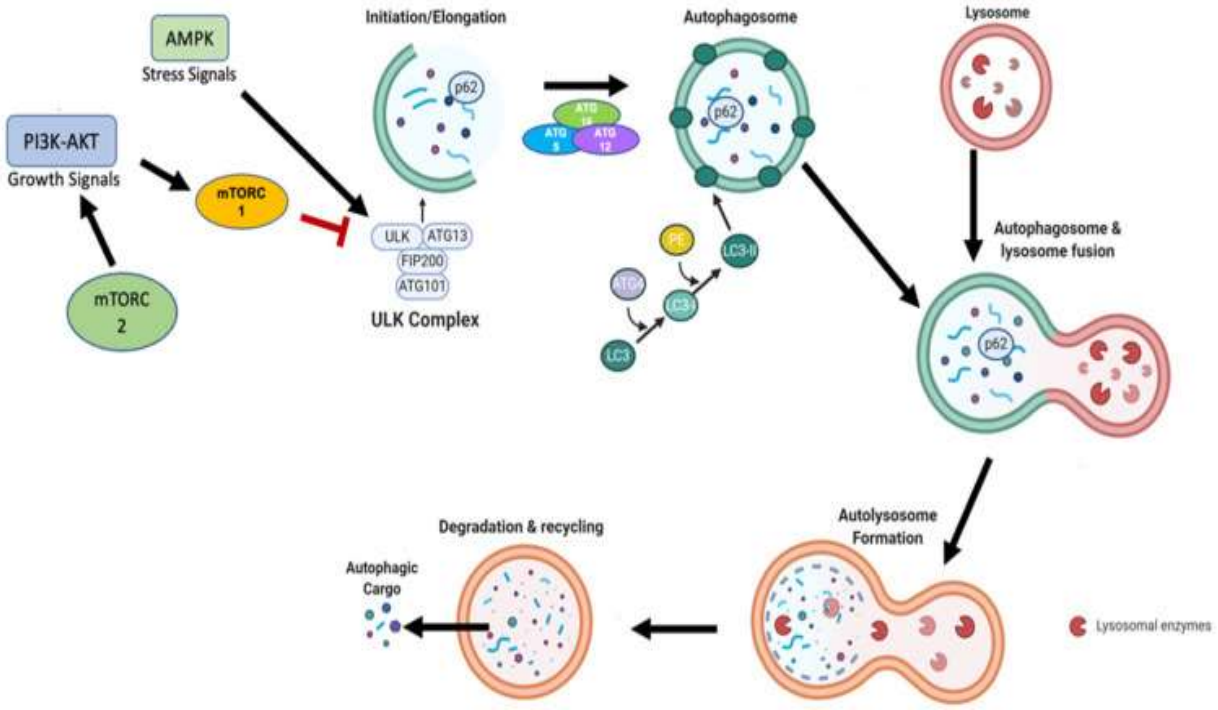


Figure 1.2. Primary mechanism of autophagy. When growth factors or nutrients become scarce, AMPK or mTOR inhibition result in activation of the ULK complex, leading to phagophore initiation. The phagophore elongates and matures with the recruitment of ATG proteins, which contribute to the formation of the phosphatidylethanolamine (PE)-Conjugated LC3-II, which incorporates into the autophagosome membrane. After fusion with the lysosome, the autophagic cargo, comprising nutrients and metabolites, is degraded in the autolysosome and recycled back into the cytoplasm.

1.3.3. Functional forms of Autophagy

Several different functional forms of autophagy have been identified in cancer progression and promotion [43], specifically cytoprotective, non-cytoprotective, cytostatic and cytotoxic autophagy. *Cytoprotective* autophagy is a survival response that enables the tumor cells to buffer against starvation and evade apoptotic signals [44]. In many cases, the induction of protective autophagy, which decreases sensitivity to chemotherapeutic drugs and radiation, is associated with drug resistance [45]. Therefore, targeting the cytoprotective form of autophagy is

considered as a potential therapeutic strategy in cancer treatment utilizing clinically-approved autophagy inhibitors such as hydroxychloroquine (HCQ).

Autophagy induction may also contribute to tumor cell killing, either of its own, or by serving as a precursor to apoptosis, the form known as *cytotoxi*c autophagy [43, 46]. The *cytostatic* form of autophagy represses tumor cell growth independent from apoptosis. This form is most likely associated with the well-characterized and prolonged growth arrest condition known as senescence, which may be also contribute to tumor delay and dormancy [43, 47].

A less well-appreciated function of autophagy is what we have termed the *non-protective* form induced by chemotherapeutic agents or radiation whose suppression does not affect cell proliferation and apoptosis. As an example, in non-small cell lung cancer cells, autophagy inhibition via pharmacological and genetic interventions did not alter the sensitivity of p53 wild type H460 cells to cisplatin [48]. Similarly, autophagy inhibition did not alter sensitivity to radiation of 4T1 breast tumor cells in cell culture, nor did chloroquine (CQ) alter the sensitivity of this cell line to radiation in an immune-competent animal model [49]. Furthermore, when non-protective autophagy is inhibited, there is no elevation in apoptotic cell death (unlike the case when cytoprotective autophagy is inhibited); however, the significance of this form of autophagy remains to be determined, since it is unclear of what advantage it might be to the cell under conditions of stress [49, 50].

1.4. Senescence

1.4.1. Senescence overview

Cellular replicative senescence was first described by Leonard Hayflick and Paul Moorhead more than five decades ago as a stable exit from the cell cycle in non-transformed fibroblasts [51, 52]. Senescence is a specialized form of growth arrest that plays a dynamic role in mediating multiple physiological and pathological processes [53]. The senescent growth arrest is stable and durable, in that, senescent cells are unresponsive to mitogenic drivers, but remain viable and metabolically active [54]. Replicative senescence represents the classical response to telomeric dysfunction that occurs due to the "end replication crisis" in dividing eukaryotic cells [55]. In addition to preventing the proliferation of cells containing dysfunctional telomeres, senescence is induced in tumor cells as a response to various treatments including cytotoxic therapies, CDK4/6 inhibition, DNA- damaging drugs or ionizing radiation [12, 56-58]. Senescence is also a well-established response to oncogene overexpression [59], thereby presenting a fundamental barrier to malignant transformation [60].

1.4.2. Hallmarks of Senescence

Aside from the durable growth arrest, which is considered the primary characteristic of senescence, senescent cells exhibit a plethora of features that collectively represent the senescent phenotype [61]. The senescence characteristics are both cell-autonomous and cell-non-autonomous [62]. Intrinsically, senescent cells exhibit an enlarged and flattened morphology, chromatin rearrangement known as senescence-associated heterochromatic foci (SAHFs) [63], and enhanced expression of the senescence-associated β-galactosidase (SA-β-gal) activity [64],

which reflects increased lysosomal content [65]. Furthermore, senescent cells are characterized by the accumulation of reactive oxygen species (ROS) coupled with ROS-mediated macromolecular damage [66]. In addition, under persistent activation of the DNA damage repair response (DDR), senescent cells display nuclear foci termed DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS); these are crucial elements that further sustain the senescent state due to the activation of DDR proteins such as p53 [67, 68]. Another essential feature defining senescent cells is the production of a diverse range of cytokines, chemokines, extracellular matrix proteases, growth factors, and other signaling molecules, collectively termed the senescence-associated secretory phenotype (SASP), which largely mediates the extrinsic effects of senescence [69, 70]. However, none of these features is uniquely representative or specific to senescence as they can also be observed in other forms of cellular stress or cell cycle arrest [61]. Accordingly, senescent cells are usually identified by the examination of a profile of multiple senescence-associated biomarkers [71].

1.4.3. Tumor Recovery and Senescence-targeted Therapies

Accelerated or premature senescence is a common tumor cell response to conventional cancer therapy including endocrine therapies [72, 73]. Senescent tumor cells are growth-arrested and exhibit the conventional hallmarks of senescence [74]. Recovery from senescence could contribute to disease recurrence, and is often associated with tumor aggressiveness and therapy resistance. Senolytics, senostatics, and senomorphics have been investigated recently for the possible elimination or at least extended suppression of dormant tumor cell populations [57, 74-77].

Senostatics do not kill senescent cells but inhibit paracrine signaling and thus block the ‘proliferation’ of senescence. Antioxidants or inhibitors of NF-κB can be efficient senostatics

[78, 79], and there is evidence that multiple flavonoids, polyphenols and other phytochemicals may have senostatic activity [80]. Senomorphics suppress senescence by suppressing SASP expression via several pathways including NF-κB, mTOR, IL-1α, and p38 MAPK [80]. In contrast, senolytic eliminate the senescent cells via targeting critical proteins involved in pro-survival and anti-apoptotic mechanisms, such as p53, p21, and Bcl-2 family proteins [80]. Our lab and others [81] have investigated the utilization of various senolytics including ABT-263 as a possible senolytic for the elimination the senescent cells that are induced by etoposide, doxorubicin, cisplatin and radiation by interfering with the interaction between BCL-XL and BAX in different tumor models [48, 57].

1.5. Epigenetic dysregulation and BET family

1.5.1 Super-enhancers

In eukaryotic cells, transcription begins with RNA polymerase binding at the gene promoters. The gene promoter regions harbor transcription initiation sites, with the transcription being regulated by various transcription factors (TFs) through binding to specific DNA sequences to recruit RNA polymerase II initiation or elongation factors. Additionally, there are some DNA sequences located near or far from promoter regions that contain multiple transcription factor binding sites referred to as “enhancers” [82]. Enhancers are genomic regions that are bound by transcription factors (TFs) and transcriptional co-activators to promote gene transcription [83]. In cancer, large clusters of these enhancers have been identified, which are referred to as super-enhancers. Super-enhancers, characterized as large clusters of enhancers in close proximity, have been identified as essential oncogenic drivers required for the maintenance

of cancer cell identity [84]. Super-enhancers are co-occupied by various TFs crucial for the relevant cell type and have occupied by high levels of transcriptional regulators, including Mediator, p300, CBP, BRD4, RNA polymerase II (RNA Pol II), cohesin, and chromatin remodelers [83]. Super-enhancers recently become an attractive target in cancer therapeutics field.

1.5.2 BET family

The bromodomain is an evolutionarily conserved protein-protein interaction module consisting of approximately 110 amino acids that can recognize and bind acetylated lysine residues in histones and many other proteins [85]. Bromodomain-containing proteins (BRDs) serve as epigenetic readers of histone acetylation, which can recruit transcriptional regulator complexes to chromatin and bind to acetylated histones [86]. Among the 8 families that contain bromodomain modules, the bromodomain and extra-terminal domain (BET) family has attracted extensive attention in the recent years [87]. The BET family of proteins is characterized by the presence of two tandem bromodomains and an extra-terminal domain. The mammalian BET family of proteins is comprised of BRD2, BRD3, BRD4, and BRDT [88], acting as epigenetic readers with broad specificity towards transcriptional activation (including the recruitment of positive transcription elongation factor [P-TEFb] and the control of RNA polymerase II [Pol II] transcriptional activity [88]. Dysregulated expression of BET family members is involved in many pathological processes and has become an important therapeutic target for various diseases, including cancer.

Beyond BRD2 [89] and BRD3[90] overexpression in different tumor models, BRD4 is the most frequently studied of the BET family members and has been shown to play critical roles in human diseases, including CNS disorders [91], cardiovascular disease [92, 93], inflammatory disease [94] as well as cancer [95, 96]. As part of the general transcription machinery, BRD4 is enriched on hyper-acetylated and transcriptionally prone chromatin regions (both promoters and enhancers) working as nucleation center, recruiting the Mediator complex and promoting the assembly of a large platform of transcription regulating proteins, that forms a bridge between SE and Promoter, favoring and stabilizing the binding of RNA-Pol II. Furthermore, BRD4 also interacts and activates P-TEFb, stimulating transition of RNA-Pol II into active elongation (**Figure 1.3**).

BRD4-occupied super-enhancers have been characterized in different types of cancers, which drive the expression of a large cohort of oncogenes to promote cancer development including c-Myc [97-99]. A large number of small-molecule inhibitors targeting BRD4 have since been developed including JQ1 , the first reported and the most studied BET family inhibitor that can bind competitively to acetyl-lysine recognition motifs or bromodomains [100], CPI203, MS417 [101] and OTX015 [102].

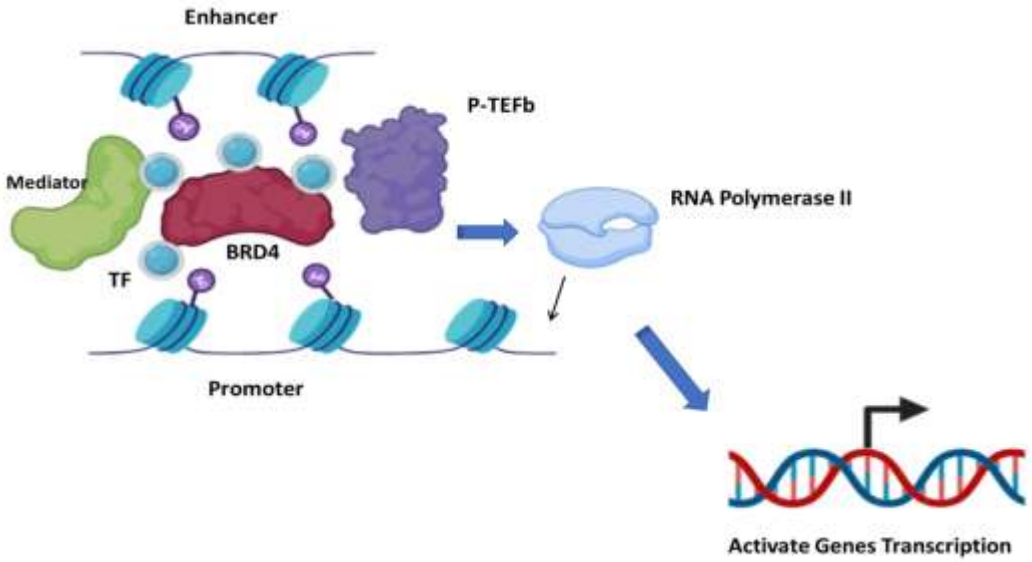


Figure 1.3. BRD4 regulation of gene transcription. BRD4 binds promoter and enhancers, forming a bridge by binding to various transcription factors and Mediator complex, supporting the binding of RNA polymerase II for transcription. Brd4 also activate P-TEFb, stimulating transition of RNA-Pol II into active elongation.

1.6. p53/p21 and growth inhibition

p53 is one of the intensely studied tumor suppressor genes that is encoded by the *P53* gene on human chromosome 17 [103]. p53 is considered the first barrier to the malignant transformation through inhibiting the phenotypic and genomic alterations associated with carcinogenesis as well as tumor development through a complex interplay with several signaling pathways known to play critical roles in essential cellular processes such as cell division, maintenance of genomic stability, apoptosis, autophagy, and the regulation of tumor microenvironment [104, 105]. p53 binding to specific DNA response elements induces the expression of a wide array of genes that ultimately guard against cancer development and progression including p21 [103]. The *CDKN1A* gene, coding for the cyclin-dependent kinase inhibitor p21/WAF1/CIP1/CDKN1A, was the first discovered transcriptional target of p53 [106,

107]. Its binding spectrum is wide, as p21 forms complexes with CDK1, CDK2, CDK3, CDK4, and CDK6 together with specific cyclins [103]. p21 has been shown to mediate p53-induced G_1 cell cycle arrest. Its induction by p53 and concomitant inhibition of CDKs is considered crucial for p21's tumor-suppressive role [103]. Several agents have been shown to mediate p53/p21 accumulation in various tumor models, inducing cellular growth arrest including 1,25-Dihydroxyvitamin D_3 [108] as well as DNA damaging agents [109].

Chapter 2: Materials and Methods

2.1. Antibodies and reagents

The following primary antibodies were used: BRD4 (Cell Signaling Technology, 13440); BRD3 (ABclonal, A16241); BRD2 (ABclonal, A2277) ; c-Myc (Cell Signaling Technology, 5605); p53 (Cell Signaling Technology, 9282) ; cleaved-Caspase 3 (Cell Signaling Technology, 9664); cleaved-PARP (Cell Signaling Technology, 9541); B-actin (Cell Signaling Technology, 4970); p21 (cell signaling, 2947) and GAPDH (Cell Signaling Technology, 2118). Secondary antibodies: Horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling, anti-mouse, 7076S; anti-rabbit, 7074S).

2.3. Cell Lines

MCF7 (Luminal A, humanly adenocarcinoma cells, p53 WT, obtained from Dr. Knudsen, Roswell Park Comprehensive Cancer Center) were cultured in DMEM (Gibco, 11995-065) supplemented with 10% (v/v) fetal bovine serum (Gemini, 26140), 100 U/mL penicillin G sodium (Invitrogen, 15140–122), and 100 μg/mL streptomycin sulfate (Invitrogen, 15140–122). T47D (Luminal A, humanely ductal carcinoma cells, p53 mutant) cells were cultured in RPMI medium (ATCC 30-2001) supplemented with 10% (v/v) fetal bovine serum (Thermo Scientific, SH30066.03), 100 U/mL penicillin G sodium (Invitrogen, 15140–122), and 100 μg/mL streptomycin sulfate (Invitrogen, 15140–122). MCF-7 p53-/- (obtained from Dr. Xinbin Chen) were cultured in RPMI medium (Gibco, 11875-093) supplemented with 10% (v/v) fetal bovine serum (Gemini, 26140), 100 U/mL penicillin G sodium (Invitrogen, 15140–122), and 100 μg/mL streptomycin sulfate (Invitrogen, 15140–122). For estrogen deprivation studies, MCF-7 were cultured in DMEM (Gibco, 21063-029) supplemented with charcoal stripped fetal bovine serum

(Gibco, A33821-01), Estradiol (Med-chem express, HY-B0141) and Insulin (Sigma, 11061-68-0).

2.4. Drug Treatment

The concentrations of the drugs being utilized in this study have been selected based on the literature as well as screening studies performed in the Dr. Gewirtz laboratory. Fulvestrant (Millipore Sigma, I4409), Palbocilib (LC Labratories, P-7788), ABT263 (AbbVie) (1uM), ABT199 (APExBio, Houston TX, USA) (1uM) , ABBV-744 (Abbive, A-1627416), Tamoxifen (Med-chem express, 121893), Bafilomycin A1 (Millipore Signma, 196000), and ARV-825 (Med-chem express, HY-16954) were dissolved in DMSO. For Fulvestrant and Palbociclib studies, cells were exposed to Fulvestrant (100 nM) plus Palbociclib (1 µM) for 6 days. For ABT-263 and ABT-199 treatment, cells were treated with Fulvestrant plus Palbociclib for 6 days and then ABT-263 (1uM) / ABT-199 (1uM) added for 48h. For BET inhibitors/degraders exposure, cells were treated with Fulvestrant plus Palbociclib for 6 days and the respective BET inhibitor/ degrader was given for 92 h post-anti-estrogen and CDK 4/6 inhibition therapy. For Tamoxifen studies, cells were exposed to Tamoxifen (5µM) for 4 days. For BET inhibitors/degraders exposure, cells were treated with Tamoxifen for 4 days and the respective BET inhibitor/ degrader was given for 92 h post-anti-estrogen therapy. For validation of the knockout by CRISPER/cas9, Cells treated with doxorubicin (cat. no. D1515; Sigma-Aldrich) at either 1.0 µM or 3.0 µM, cells were then, ultimately harvested after 24 h.

2.5. Organoid cell culture

The MCF7 and T47D cell lines were seeded in 96-well dish (4000 cells/well) that were precoated with 50% Matrigel (Corning; 354234). Cells were allowed to form organoids up to 48

H and were treated with the experimental drugs. The viability of organoids was determined using Cell-Titer Glo 3D cell viability assay kit (Promega).

2.6. Cell Viability

Trypan blue exclusion was utilized to assess cell viability. Cells were plated at 20,000 cells per well in a 6-well plate and treated with the respective conditions. On the indicated days, cells were trypsinized, stained with 0.4% trypan blue (Sigma, T01282), and counted on the indicated days using a hemocytometer. Growth curves were generated from the collected data.

For MTS assay, cells were plated in 96 wells plate, in a suitable concentration, treated under the indicated conditions. The viability was assessed by UV spectrophotometer using MTS reagent (AB197010).

2.7. LDH assay

MCF-7 cells were plated in 96 wells plate, 200 cells per well, treated with different concentrations of ABBV-744 (25, 50, 75 and 100 uM) for four days. LDH assay was performed using CyQUANT LDH assay Cytotoxicity Assay Kit (Invitrogen, C20300 and C20301) according to the manufacturer's protocol.

2.8. Clonogenic survival assay

Cells were plated at a density of 200 cells per well in 6-well plates and treated with the respective conditions. Media was replenished every other day until colonies form. Colonies were washed with 1X phosphate-saline buffer (PBS, Life Technologies), fixed with 100% methanol and stained with 0.1% crystal violet (Sigma). The number of colonies formed were counted.

2.9. Promotion of Apoptosis

The extent of apoptotic cell death was measured using Annexin V-FITC/Propidium iodide staining. On the indicated day, cells were trypsinized, washed with 1X PBS and stained according to manufacturer protocol (Annexin V-FITC Apoptosis Detection Kit; BD Biosciences, 556547). Fluorescence was quantified by flow cytometry using BD FACSCanto II and BD FACSDiva software at the Flow Cytometry Core Facility at Virginia Commonwealth University. For all flow cytometry experiments, 10,000 cells per replicate were analyzed and three replicates for each condition were analyzed per independent experiment unless otherwise stated. All experimental protocols were performed with cells protected from light.

2.10. SA-ß-gal staining

On the indicated days, cells were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining as previously described by Dimri et al [64]. Cells were washed with 1X PBS and phase contrast images were taken using an inverted microscope (Olympus, Tokyo, Japan).

MCF-7 wild type cells were treated with the respective conditions. The cells were stained for β-galactosidase to determine the senescence phenotype by using the commercially available kit (Abcam; ab65351) according to the manufacturer's protocol. Cell images were taken using phase-contrast microscope at 20X magnification.

To quantify ß-gal positive senescent cells, after treatment, cells were treated with Bafilomycin A1 (100 nM) for 1 h to achieve lysosomal alkalinization, followed by staining with C12FDG (10 μM) for 1 h at 37 ℃. After incubation, cells were collected and analyzed by BD FACSCanto II and BD FACSDiva software. For all flow cytometry experiments, 10,000 cells

per replicate were analyzed and three replicates for each condition were analyzed per independent experiment unless otherwise stated. All experimental protocols were performed with cells protected from light.

2.11. Western blot analysis

Western blotting was performed as previously described 20 [110]. In brief, after indicated treatments, cells were trypsinized, harvested, and washed with 1X PBS. Pellets were lysed and protein concentrations were determined by the Bradford Assay (Bio-Rad Laboratories, 5000205). Protein samples were loaded and subjected to SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and blocked with 5% milk in 1X PBS with 0.1% Tween 20 (Fisher, BP337). The membrane was incubated overnight at 4°C with the indicated primary antibodies at a dilution of 1:1000 in 5% BSA. The membrane was then washed, secondary antibody was added at a dilution of 1:2000 in 5% BSA for 2h at room, and the membrane was washed again with 1X PBS with 0.1% Tween 20 three times. Blots were developed using Pierce enhanced chemiluminescence reagents (Thermo Scientific, 32132) on BioRad ChemiDoc System. Quantification was done using Image J software.

2.12. Immunoprecipitation

p53 (Santa Cruz, DO-1) or BRD4 (Cell Signaling, E2A7X) primary antibodies (1:100 dilution) were added to equal amounts of whole cell lysates and incubated with rotation at 4^{o}C overnight. Antibody complexes were then captured using Protein A/G UltraLink Resin (Thermo Fisher, 53132) at 4^{o}C with rotation for 1 hour. Samples were centrifuged, washed with CHAPS buffer 3x, and resuspended in CHAPS buffer and 5x-SDS loading buffer. After boiling the samples for 5 minutes, they were analyzed by western blotting as already described.

2.13. qRT-PCR

Fulvestrant + Palbociclib treated cells were harvested at Day 6 after drug treatment, and total RNA was extracted using RNeasy kit (QIAGEN, Germany) following the manufacturer's instructions. cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad, USA) based on the protocol that manufacture provided. cDNAs from different samples were amplified in technical triplicates using iTaq Universal SYBR® Green Supermix from BioRad in QuantStudio™ 3 Real-Time PCR System (Thermofisher, USA). QuantiTect primers were purchased from Qiagen: CXCL8: QT0000322; IL-6: QT00083720; IL-1β: QT00021385; MMP3: QT00060025; GAPDH: QT00079247. Relative mRNA expression was determined using the ΔΔCt method.

2.14. Cell proliferation assay

To determine the proliferation of T47D cells in real time, live cell imaging using IncuCyte S3 was performed. Cells were seeded in 96 well dish (1000 cells/well) and allowed to adhere overnight. The cells were exposed to Palbociclib (1 µM) in combination with Fulvestrant (100 nM) for 6 days and the cell division as monitored using IncuCyte that performs nuclei count. Following 6 days, the cells were released from the Palbociclib/Fulvestrant combination treatment and allowed to grow in the absence and presence of ARV825 (50 nM) for 96 H. Following 96H exposure, the cells were released from ARV825, and the cellular outgrowth was monitored. Based on the nuclei counts, the relative proliferation rate was determined. Growth cures were generated using GraphPad Prism.

2.15. Statistics

Unless otherwise indicated, all quantitative data is shown as mean ± SEM from at least three independent experiments, all of which were conducted in triplicates or duplicates. GraphPad Prism 9.0 software was used for statistical analysis. All data was analyzed using either a one- or two-way ANOVA, as appropriate, with Tukey or Sidak post hoc.

Chapter 3: Autophagy inhibition and BRD4 targeting as possible strategies to extend the growth arrest mediated by Fulvestrant + Palbociclib in ER-positive breast cancer.

3.1. Introduction

Breast cancer is the most commonly occurring malignancy in women [1, 111], and the second most common cause of cancer-related deaths, with approximately 40,000 women dying from breast cancer each year in the U.S [2, 111]. Among the various types of breast cancer, estrogen receptor alpha positive (ER+) breast cancer is the most common form, constituting approximately 70% of all breast cancer cases [8, 112]. The standard of care for this breast cancer subtype is endocrine therapy in combination with adjuvant therapy, which has reduced relapse and mortality by up to 40% [6]. The clinically available endocrine therapies include selective estrogen receptor modulators (SERMS) such as Tamoxifen (TAM), selective estrogen receptor degraders (SERDs), such as Fulvestrant [7], and aromatase inhibitors (AIs) such as letrozole [8]. The current standard of care for metastatic ER-positive breast cancer utilizes the combination of either the estrogen receptor degrader Fulvestrant or aromatase inhibitors such as Letrozole with CDK4/6 inhibitors such as Palbociclib, Ribociclib and Abemaciclib. The combination of Letrozole with Palbociclib as an initial therapy has extended progression free survival in advanced ER+ HER2- breast cancer from 14.5 months to 27.6 months [113] . Once the disease progressed on prior endocrine therapy, the combination of Fulvestrant with Palbociclib extended progression-free survival in breast cancer patients from 4.6 to 11.2 months [113]; however, escape from the growth suppressive effects of this combination is quite common, leading to disease relapse.

3.1.1. Autophagy in response to Fulvestrant and Palbociclib in ER+ breast cancer

The role of autophagy in Fulvestrant resistance is not well detailed. There are a very limited number of studies that have investigated the relationship between Fulvestrant resistance and autophagy with a controversial results *in vitro* and *in vivo*. For example, Cook et al. [114] did utilize Fulvestrant as a single agent and in combination with HCQ for both *in vivo* and *in vitro* experiments conducted with the MCF-7 cell line. For the *in vitro* studies, treatment with Fulvestrant resulted in increased LC3-II with p62/SQSTM1 degradation, confirming autophagy induction. The combined treatment of HCQ and Fulvestrant resulted in a significant reduction in MCF-7 cell viability as compared to Fulvestrant used as a single agent. Autophagy inhibition by HCQ was demonstrated by the accumulation of LC3-II and p62/SQSTM1 in MCF-7 cells [114]. However, the *in vivo* studies performed by Cook et. al. [114] did not recapitulate the *in vitro* results. The combination of Fulvestrant and HCQ was less effective than HCQ treatment alone, while Fulvestrant used as a single agent showed no difference in the tumor size as compared to the controls.

As the case with Fulvestrant, a limited number of studies suggest that autophagy contributes to ER+ breast cancer cell survival when Palbociclib is used as a single agent, but to a much lesser extent when used in combination with hormonal treatments. Studies by Vijayaraghavan et al. [12] have demonstrated that Palbociclib induces autophagy in MCF7 and T47D breast cancer cell lines, as indicated by increased MDC staining, autophagosome generation, and increased levels of LC3B-II, Atg-7, Beclin-1, BNIP3, as well as p62 reduction [12]. While Beclin-1 or Atg-5 knockdown alone showed no effect on cell viability, when combined with Palbociclib, these genetic approaches for autophagy suppression significantly

increased MCF7 and T47D cell sensitivity to Palbociclib. Interestingly, the suppression of autophagy was accompanied by increased senescence. In support of these findings, Palbociclib in combination with HCQ resulted in enhanced growth inhibition as well as increased cellular senescence compared to Palbociclib alone, without inducing apoptosis [12].

In vivo studies involving mice orthotopic xenografts of MCF7 breast cancer cells treated with Palbociclib demonstrated a significant reduction of the tumor volume and autophagy induction. Elevated levels of Atg-7 and increased degradation (turnover) of LC3B-II and p62/SQSTM1 were detected, along with increased autophagosome production in tumor cells. Importantly, treatment with the combination of Palbociclib and HCQ resulted in significantly smaller tumor volumes than for Palbociclib alone. Another autophagy inhibitor, Lys05, used *in vivo* in combination with Palbociclib, generated a similar trend to the studies utilizing HCQ with smaller tumors and prolonged survival compared to the controls [12]. These results are consistent with a cytoprotective role for Palbociclib induced autophagy. However, no publication is available on using Fulvestrant in combination with Palbociclib, which would be more clinically relevant. Furthermore, it is important to note that autophagy role is variable and dependent on the cell line as well as the chemical nature of the compound being used.

3.1.2. BET inhibition as a therapeutic strategy and its association with senescence

There has been growing interest in the potential targeting of dysregulated epigenetic regulation, such as “super enhancers” in cancer treatment [115]. Super enhancers are clusters of enhancers with unusually high levels of transcription factor binding, which are central to driving elevated oncogenic transcription [116]. The Bromodomain and extra terminal (BET) protein

family, including BRD2, BRD3, and BRD4, by binding to acetylated lysine residues on histone proteins, epigenetically regulate the transcription of various genes and can interact with super enhancers [117]. BET inhibition has demonstrated efficacy in pre-clinical studies and is being evaluated in various clinical trials for both hematological malignancies and solid tumors [118]. Among the different BET inhibitors that have been investigated and showed promising preliminary results are JQ1 [100], CPI203, MS417 [101] and OTX015 [102].

Therapy induced senescence is a form of growth arrest which has been shown to contribute to chemotherapy resistance [72, 119]. Recent studies have investigated the possible utilization of BET inhibitors as senolytics, such as in the work by Wakita et al. who have shown that ARV825 and JQ1 have a possible senolytic activity using different cancer models [120].

3.1.3. Overarching hypotheses

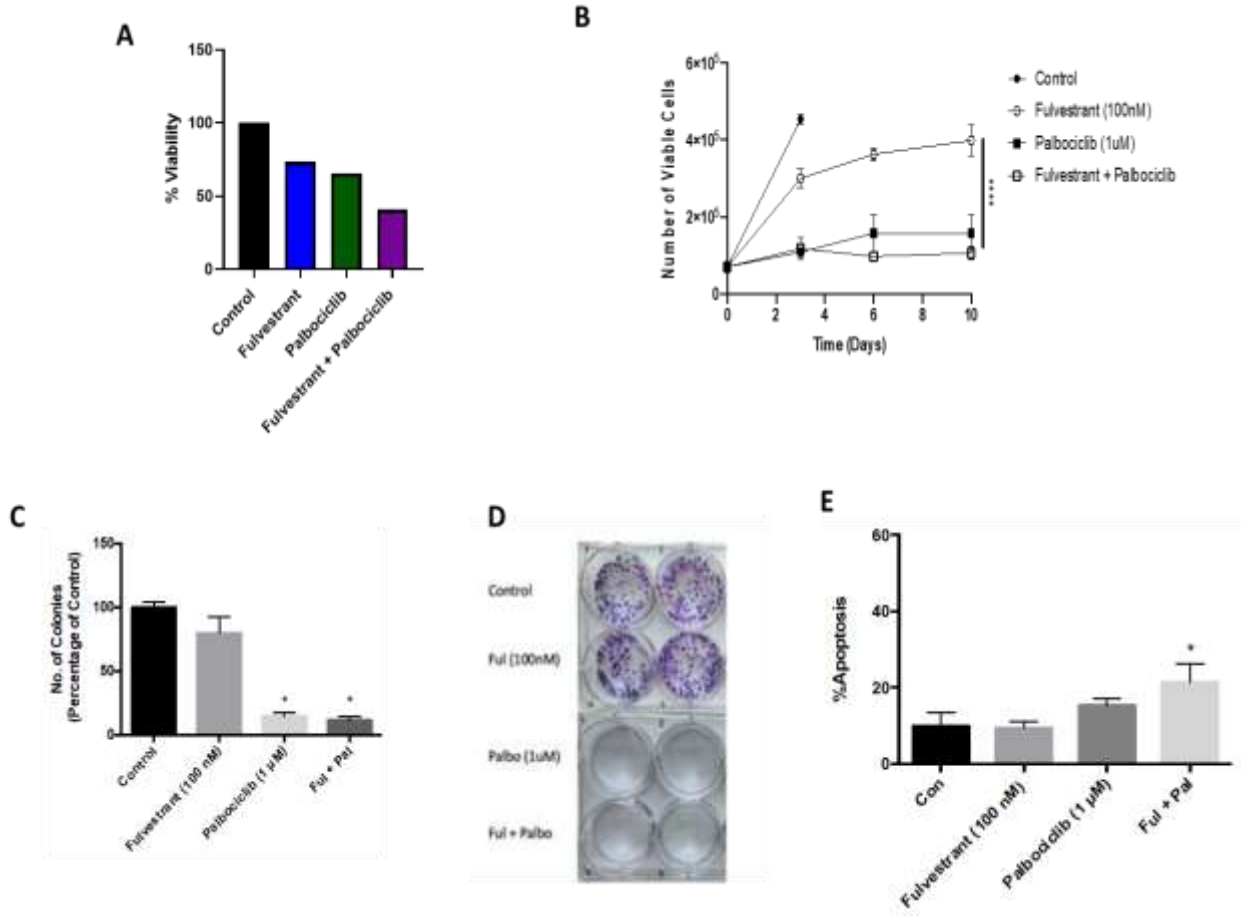
Different treatment modalities including chemotherapy and radiation have been shown to contribute to resistance development for endocrine therapies by autophagy and/or senescence induction. Consequently, we hypothesized that either autophagy inhibition or senescent cell elimination could be possible strategies to increase the effectiveness of one of the current standards of care therapy for ER+ breast cancer, Fulvestrant plus Palbociclib.

3.2. Results

3.2.1. The growth arrest response to Fulvestrant + Palbociclib in MCF7 breast tumor cells

While anti-estrogen therapy combined with CDK 4/6 inhibition is the standard of care for ER+ breast cancer patients diagnosed with locally advanced or metastatic disease progression following endocrine therapy, this combination therapy only modestly prolongs patient survival [121]. In an effort to simulate the clinical treatment regimen in an *in vitro* environment, MCF7 cells were exposed to Fulvestrant and Palbociclib for 6 days and fresh media was replenished after drug removal on day 6. Cell viability was assessed utilizing MTS assay as well as trypan blue exclusion on the indicated days (**Figure 3.1. A and B**). Fulvestrant initially delayed tumor cell growth and, after a delay, arrested the cells. Palbociclib, alone, and in combination with Fulvestrant, completely halted the growth of the MCF7 cells; however, the cells generally began to recover from treatment after a period of approximately 8-12 days (**see Figures 3.6**).

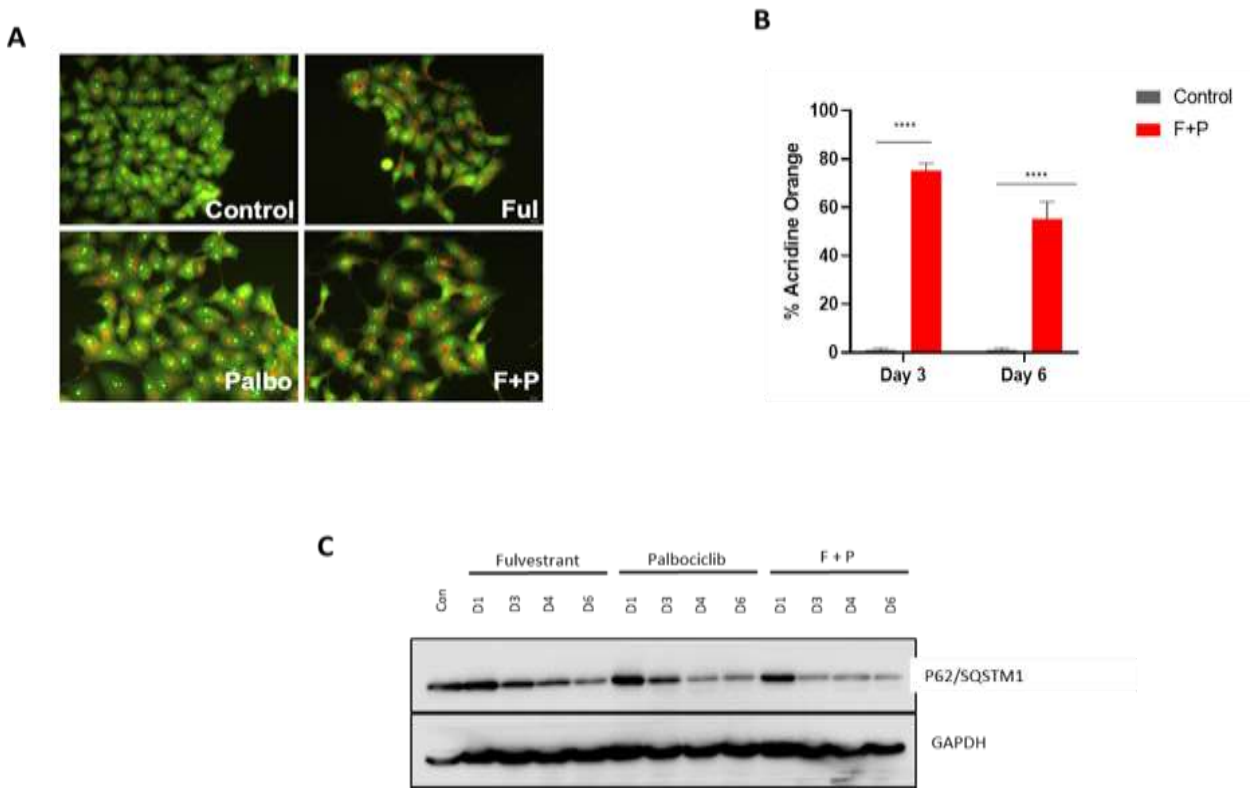
The effectiveness of Palbociclib alone as well as the combination treatment in suppressing cell growth was confirmed by clonogenic survival studies (**Figure 3.1. C and D**). The growth inhibitory effect of Fulvestrant alone did not achieve significance, in contrast to the moderate effects in the temporal response assay; consequently, the observed effects are largely Palbociclib driven. While therapy-induced tumor cell death is the desired outcome of anti-cancer treatment, there was a relatively low degree of apoptosis in MCF7 cells treated with Fulvestrant, Palbociclib or the combination (**Figure 1E**), which may be permissive for proliferative recovery. Consequently, one of the primary goals of the present work was to identify a strategy that might convert the growth arrest response to one of cell death, initially through efforts to block autophagy.



3.1. Fulvestrant in combination with Palbociclib in MCF7 cells. Cells were treated with Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days (A-E). (A) Screening the effect of each treatment condition using MTS assay. (B)(#)Viability of MCF7 cells was monitored based on trypan blue exclusion at indicated days following drug exposure (n=3). ****P ≤ 0.0001 indicate statistical significance of each condition compared to Fulvestrant alone. (C) (#) After exposure for 6 days, cells were incubated in fresh medium for 7 days. Quantification of colonies expressed as relative percentage compared to controls (n=3). (D) (#) Representative colony formation 7 days after drug removal by crystal violet staining. (E) (#) Apoptosis was measured using annexin V/PI staining at the end of the 6-day treatment and fluorescence was measured using flow cytometry (n=3). Unless stated otherwise, data were from three independent experiments. *P ≤ 0.05 indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak's post hoc test.

3.2.2. Fulvestrant and Palbociclib-induced autophagy

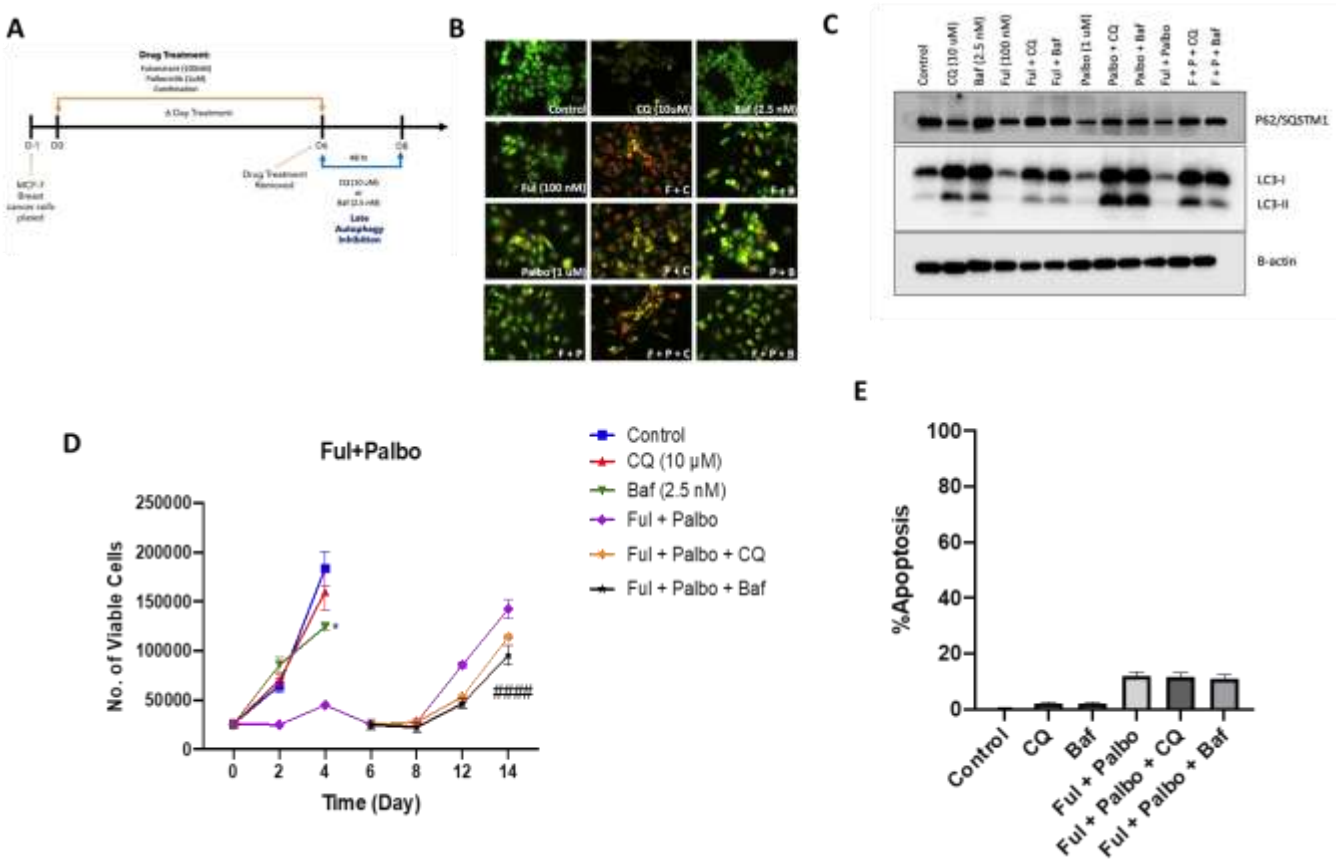
In response to therapy, cancer cells upregulate multiple mechanisms in attempts to evade cell death, one of which is autophagy [8, 43, 122]. Autophagy is conventionally considered to be a cytoprotective process that allows cells to combat either intrinsic or extrinsic forms of injury; however, other functions of autophagy have been identified, specifically a cytotoxic form [8, 123] and what has been termed as a non-protective form [8, 43, 124, 125]. Consequently, we examined whether autophagy was induced in response to the anti-estrogen, Fulvestrant, and CDK4/6 inhibition therapy in MCF7 cells. Initially, acridine orange was utilized at day 4 to assess acidic vesicle formation. **Figure 3.2.A** demonstrates basal autophagy in these cells as well as increased acidic vesicle generation in response to Fulvestrant or/and Palbociclib. As our interest is in the combination of Fulvestrant plus Palbociclib, **Figure 3.2.B** further provides quantification of fluorescence via flow cytometry and indicates that autophagy is substantially induced at both day 3 and day 6. To verify autophagy induction, western blot analysis in **Figure 3.2.C** revealed a temporal decline in p62/SQSTM1 levels in MCF7 cells treated with Fulvestrant, Palbociclib and, most importantly, the drug combination.



3.2. Fulvestrant in combination with Palbociclib induces autophagy. Cells were treated with Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days (A–C). Lysates were collected at specified days (A) (#) Cells were stained with acridine orange on day 4 and imaged using a fluorescent microscope. All images were taken at the same magnification (scale bar= 200 µm, n=3). (B) (#) Cells were stained with acridine orange and fluorescence was quantified using flow cytometry. (C) Autophagy induction over time was assessed by degradation of p62/SQSTM1 protein levels. All images are representative fields or blots from at least three independent experiments. ****P ≤ 0.001 indicate statistical significance of each condition compared to the control as determined using two-way ANOVA with Sidak's post hoc test.

3.3. Efforts to sensitize MCF-7 breast tumor cells *via* autophagy inhibition

In an effort to sensitize MCF7 cells to the Fulvestrant plus Palbociclib, the lysosomotropic agents, chloroquine (CQ) and bafilomycin A1 (Baf), were utilized as pharmacological inhibitors of autophagy, based on their ability to interfere with autophagosome-lysosome fusion [126]. Consequently, MCF7 cells were treated with Fulvestrant, Palbociclib or the combination for 6 days, and then exposed to CQ or Baf for 48 hrs post treatment (**Figure 3.3.A**). Autophagy inhibition has been confirmed based on the interference with lysosomal acidification by acridine orange staining (**Figure 3.3.B**). Autophagy inhibition by Baf and CQ was additionally confirmed by western blot analysis of LC3-II and p62/SQSTM1 , in which, we observed an increase in LC3-II accumulation and accumulation of p62/SQTM1 (interference with degradation) with CQ and Baf (**Figure 3.3.C**). **Figure 3.3.D** indicate that the addition of either CQ or Baf only very slightly slighty sensitizes MCF-7 cells to the combination treatment. Furthermore, there was minimal promotion of apoptosis with autophagy inhibitin (**Figure 3.3.E**).



3.3. Autophagy inhibitiononly slightly affecst the sensitivity to Fulvestrant plus Palbociclib in MCF7 cells. (A) Schematic of *in vitro* treatment. Cells were treated with Fulvestrant (100 nM), Palbociclib (1 µM) or the combination for 6 days, drugs were removed, and cells were given an additional 48 h CQ (10 uM) or Baf A1 (2.5 nM). (B) (#) Cells were stained with acridine orange on day 8 and imaged using a fluorescent microscope. All images were taken at the same magnification (scale bar= 200 µm, n=3). (C) Autophagy inhibition was confirmed by western blot analysis assessing accumulation of LC3 I-II and p62/SQSTM1 protein levels. (D)(#) Viable cell number was counted via trypan blue exclusion on the indicated days. (E) (#) Apoptosis was measured using annexin V/PI staining. Staining was performed on day 8 and fluorescence was measured using flow cytometry. All images are representative fields, blots, or data from at least three independent experiments. *P ≤ 0.05, and ###P ≤ 0.001, indicate statistical significance of each condition compared to Fulvestrant, Palbociclib or the combination of Fulvestrant and Palbociclib as determined using two-way ANOVA with Sidak's post hoc test.

3.2.4. Efforts to sensitize T47D breast tumor cells *via* autophagy inhibition

Approximately 20% of ER positive breast cancers present with p53 mutations [127]. In order to evaluate whether autophagy inhibition would also be effective against p53 mutant ER+ breast tumors treated with the Fulvestrant + Palbociclib combination, we assessed the number of viable cells at day 8 using the MTS cell viability assay. T47D cells were treated with Fulvestrant + Palbociclib for 6 days followed by 48-hour exposure to CQ or BAF A1 (**Figure 3.4.A).** Autophagy inhibition failed to sensitize T47D cells to the Fulvestrant + Palbociclib combination treatment (**Figure 3.4.B**). These results toghther with the data in MCF-7 **(Figure 3.3)** indicate that autophagy-induced by Fulvestrant plus Palbociclib was functionally nonprotective. We observed an increase in accumulation of LC3-II in the presence of CQ and Baf in both control and drug treated groups, indicating that CQ and Baf inhibited both basal and treatment-induced autophagy in T47D cells (**Figure 3.4.C**).

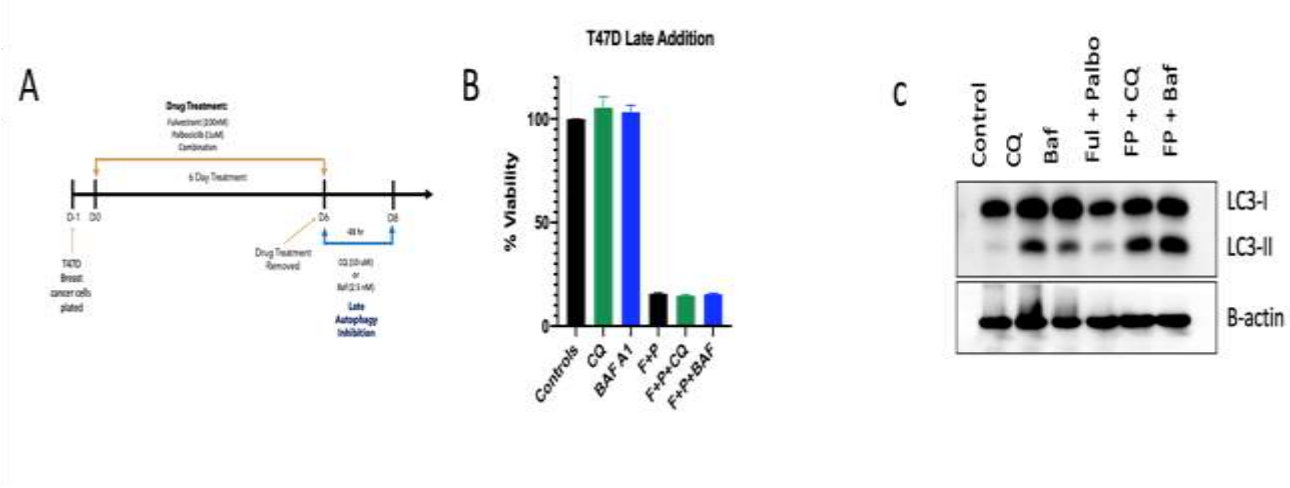
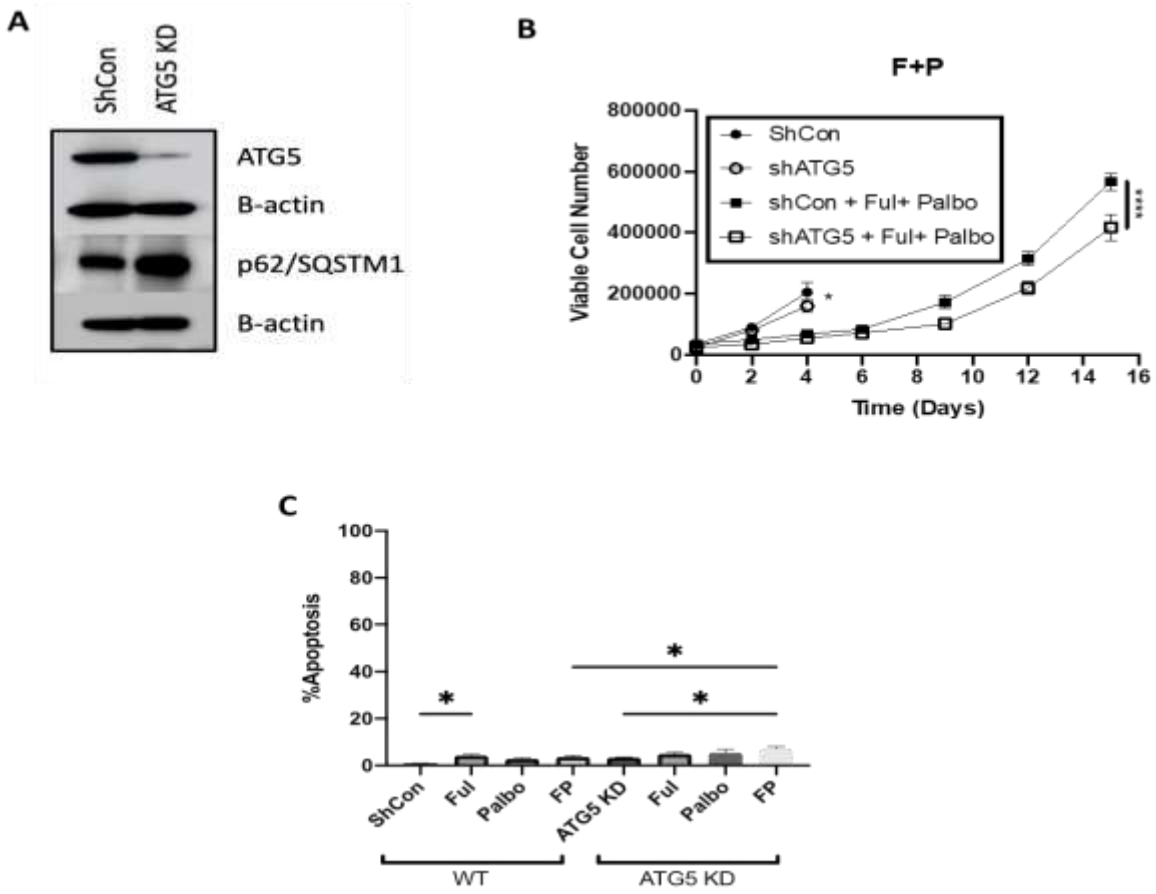


Figure 3.4. Autophagy inhibition does not alter sensitivity to Fulvestrant in combination with Palbociclib in T47D cells. T47D cells were treated with Fulvestrant (100nM), Palbociclib (1 μM) or the combination for 6 days, drugs were removed, and cells were given an additional 48h CQ (10 uM) or Baf A1 (2.5 nM). (A) Schematic of *in vitro* treatment of the addition of CQ and Baf. (B) Percent cell viability was measured after 48h of CQ and BAF addition usin**g** MTS viability assay. (C) Western blot analysis after 48h of CQ and BAF addition assessing accumulation of LC3 I-II. All images are representative fields or blots from at least two/three independent experiments.

3.2.5. Efforts to sensitize MCF-7 breast tumor cells by autophagy inhibition via genetic silencing.

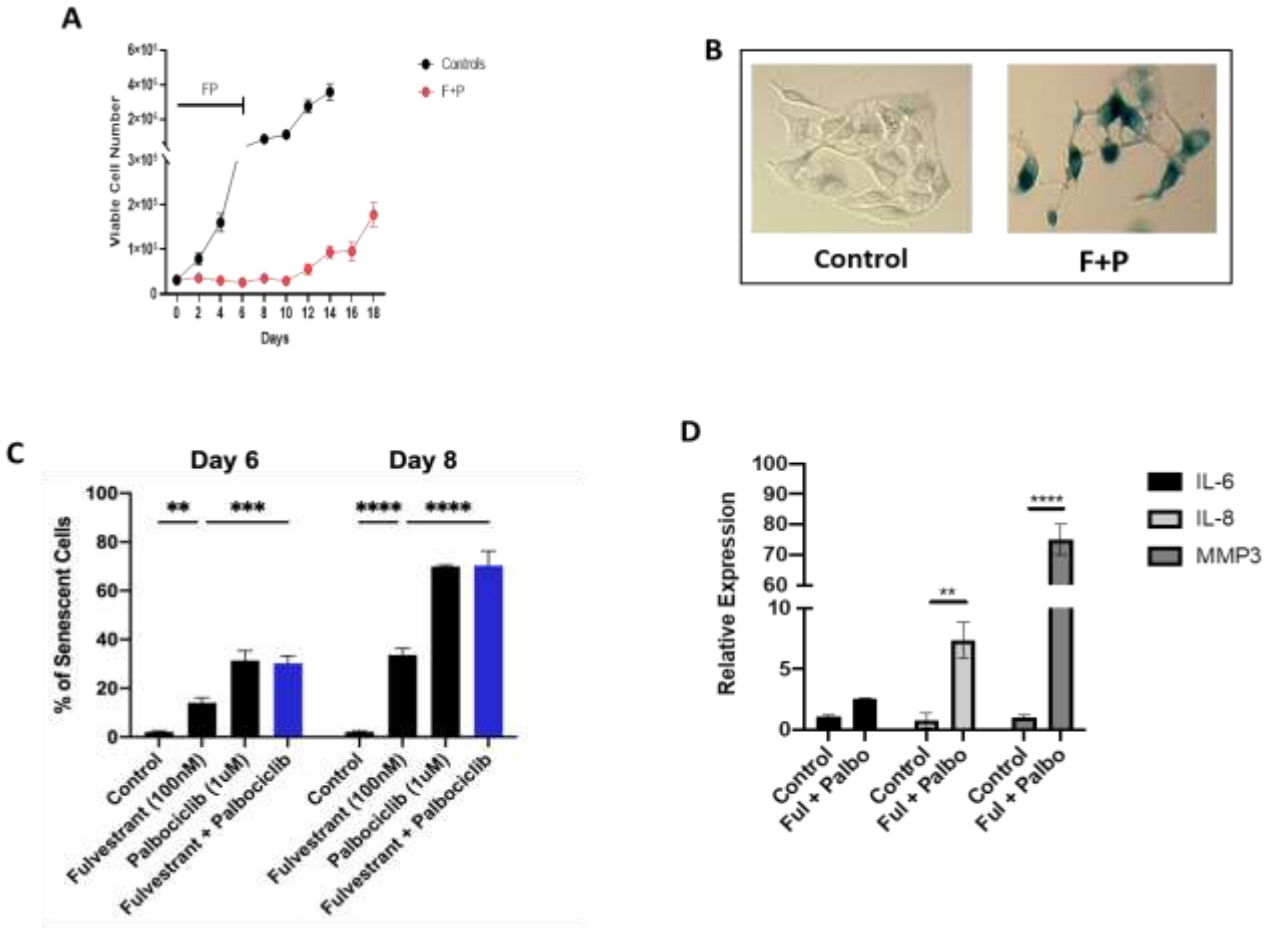
To further confirm the absence of a pronounced sensitization to the combination treatment of Fulvestrant plus Palbociclib *via* autophagy inhibition, the MCF-7 cells were stably transfected using short hairpin RNA for ATG5 (shATG5) or scrambled control (shControl). Knockdown of ATG5 and impairment of autophagy was confirmed by western blot analysis indicating reduced levels of ATG5 and accumulation of p62/SQSTM1 (**Figure 3.5.A**). Temporal analysis of cell viability showed that the MCF7 shATG5 cells were only slightly more sensitive to the Palbociclib + Fulvestrant combination therapy when compared to shControl cells (**Figure 3.5.B**). These data with genetic knockdown of ATG5 are consistent with the observed outcomes upon pharmacological inhibition of autophagy with CQ and Baf (**Figure 3.3**). Genetic silencing of autophagy also did not promote apoptosis in MCF-7 cells when exposed to Ful or Pablo, but exhibited statistically significant, albeit minimal apoptotic cell death with the combination treatment compared to shControl MCF7 cells (**Figure 3.5.C**). Taken together, these studies indicate that the autophagy induced by the combination of Fulvestrant plus is largely non-protective, and suggests the possibility that autophagy inhibition may not prove to be an effective strategy to enhance the therapeutic response [128, 129].



3.5. Genetic knockdown of autophagy only moderately increases sensitivity to Fulvestrant in combination with Palbociclib in MCF7 cells. Cells were treated with Fulvestrant (100 nM), Palbociclib (1 μM) or the combination for 6 days (A–C). Short hairpin RNA was used to knockdown ATG5. (A) Western blot analysis of ATG5 and p62/SQSTM1 protein levels. (B)(#) Viable cell number was counted via trypan blue exclusion on the indicated days. (C)(#) Apoptosis was measured using annexin V/PI staining. Staining was performed on day 8 and fluorescence was measured using flow cytometry. Unless stated, otherwise data were from three independent experiments. *P ≤ 0.05 and ****P ≤ 0.0001 compared to shControl cells treated with Fulvestrant, Palbociclib or the combination of Fulvestrant and Palbociclib.

3.2.6. Fulvestrant plus Palbociclib induce senescence in MCF-7 cells.

Given that administration of Fulvestrant + Palbociclib either alone or in combination, induces a transient growth arrest, and that autophagy and senescence tend to occur in parallel, we examined senescence induction, a durable growth arrest induced by therapy [13, 110, 130, 131]. Previous work from our group and others has consistently shown proliferative recovery from various models of therapy induced senescence [48, 57, 132-135]. The combination of Fulvestrant plus Palbociclib drives cancer cells into a state of senescence, from which the cells escape between days 12-18 **(Figure 3.6.A).** Senescence induction is further confirmed using ß-galactosidase staining, flow cytometry quantification of C_{12}FDG (a metabolite for ß-galactosidase) fluorescence, as well as assessing the expression of senescence associated secretory phenotype (SASP), IL-6,IL-8 and MMP3, using PCR **(Figure 3.6.B-D).**



3.6. Fulvestrant in combination with Palbociclib induced senescence in MCF-7 cells. Cells were treated with Fulvestrant (100nM), Palbociclib (1uM) or the combination for 6 days. (A) Cells were treated with Fulvestrant (100nM) plus Palbociclib (1uM) for 6 days and cell viability was monitored over a period of 18 days by trypan blue exclusion. (B) Cells were fixed on Day 6, stained with x-gal staining solution and imaged using bright field microscope. All images were taken with the same magnification. (C)(#) Quantification of SA-Bgal using C12FDG at indicated timepoints. (D)(#) qRTPCR examining SASP mRNA expression of IL-6, IL-8, and MMP3 on D6 post-combination treatment. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak's post hoc test. All images are representative fields, blots, or data from three independent experiments (n = 3).

3.2.7. ABT-199 and ABT-263 are ineffective in eliminating the senescent population induced by Fulvestrant plus Palbociclib

Given the promising results in eliminating the senescent populations as a potential strategy in cancer therapeutic field [48, 57], one of the senolysis mechanisms is targeting the anti-apoptotic Bcl_2 family proteins including Bcl-xl and Bcl_2 that may be upregulated as a resistance mechanism in response to various chemotherapy[136]. ABT-199 has been proven to as effective Bcl-2 inhibitor which has shown promising results in non-Hodgkin's lymphoma (NHL) [137], CLL [138], and acute leukemias [139, 140] *in vitro*. *In vivo* mouse xenograft studies showed activity against aggressive (Myc+) lymphomas [141] as well as acute leukemia [142]. Consequently, we tested ABT-199 effect on the senescence state induced by Fulvestrant plus Palbociclib. **Figure 3.7.A** showed that no significant reduction occurred in the number of viable cells in treated groups for over 12 days, and the cells appeared to recover quickly independent of ABT-199. We also investigated the effect of another BCL2 family protein inhibitor, ABT-263, which shown senolytic activity in different tumor models [57, 119]. However, ABT-263 showed the same pattern as ABT-199 with no effect on the viability of the cells treated with Fulvestrant plus Palbociclib (**Figure 3.7.B**). Consistent with these outcomes, we confirmed there was no change in Bcl-xl protein expression at days 2,4 and 6 of the treatment with Fulvestrant +Palbociclib (**Figure 3.7.C)**, which could explain why BCL inhibition is not effective strategy.

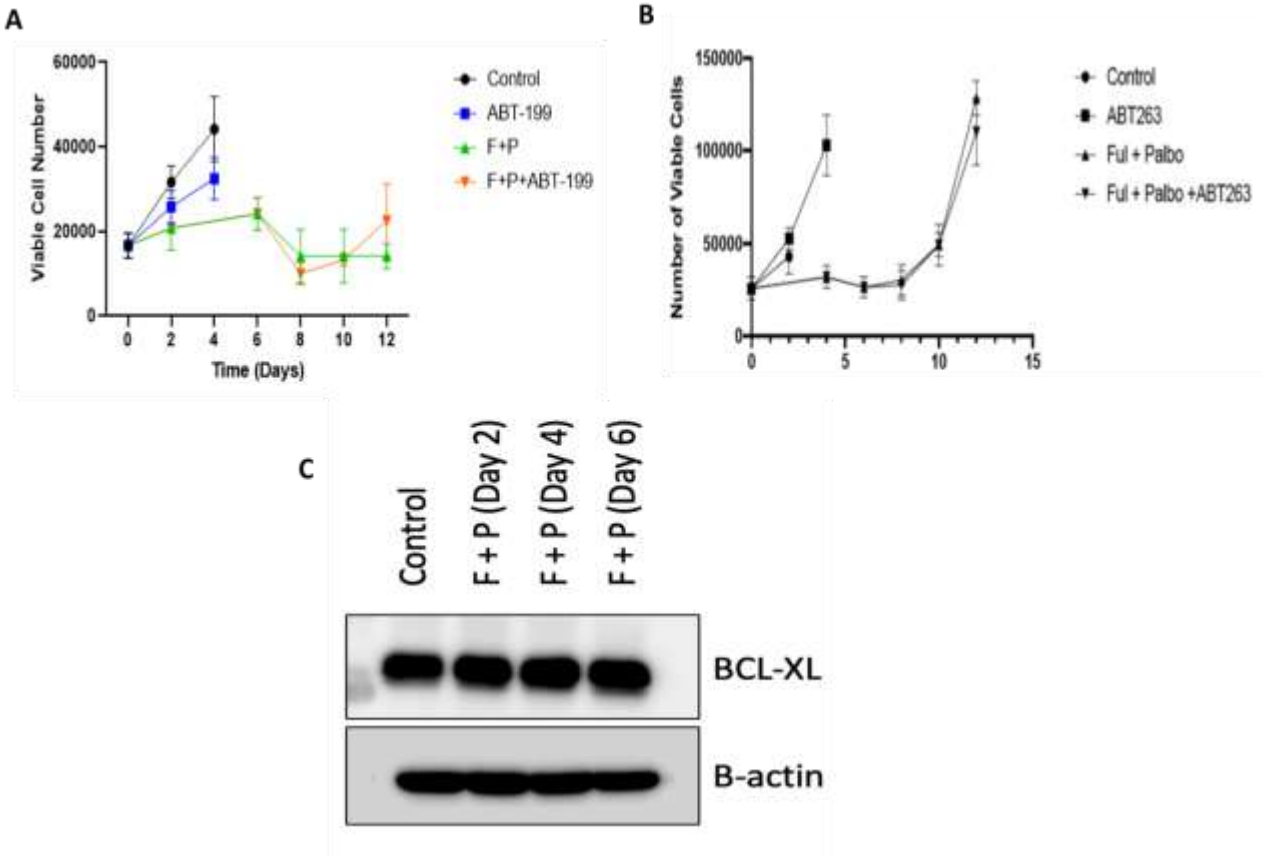
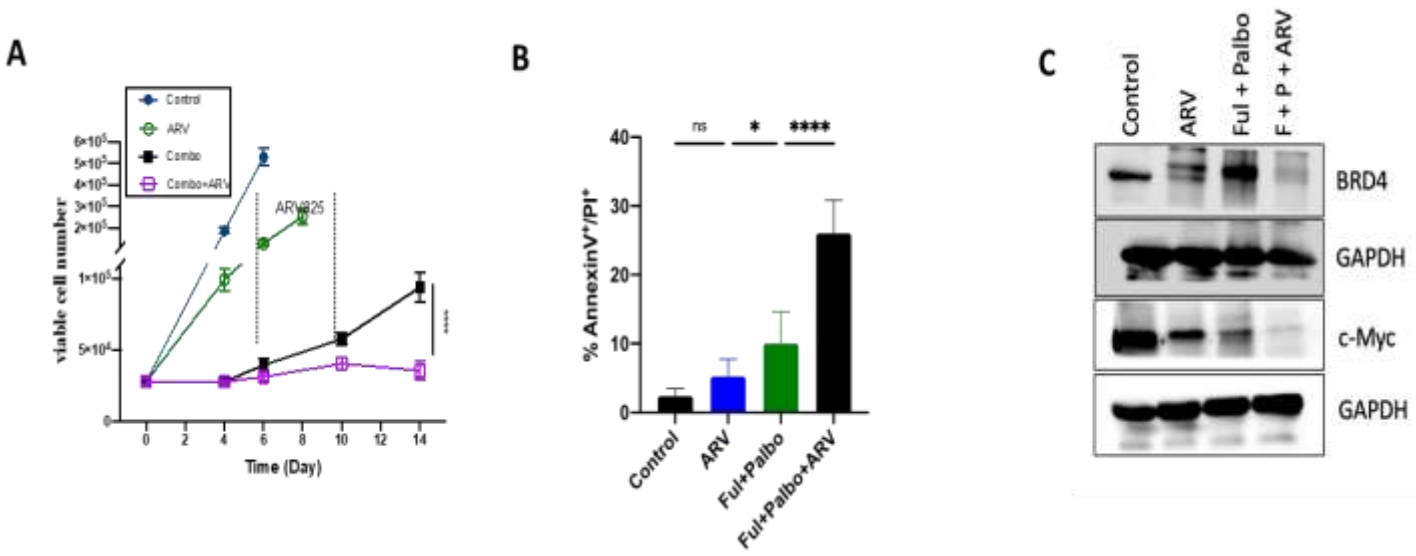


Figure 3.7. ABT-199 and ABT-263 have no impact on cell viability after Fulvestrant + Palbociclib treatment in MCF7 cells. (A) Cells were treated with the Fulvestrant (100 nM) + Palbociclib (1 µM) combination for 6 days, followed by drug removal and the addition of ABT-199 (1uM) for 48 hours and the viable cell number was counted via trypan blue exclusion on the indicated days. (B)(#) Cells were treated with the Fulvestrant (100 nM) + Palbociclib (1 µM) combination for 6 days, followed by drug removal and the addition of ABT-263 (1uM) for 48 hours and viable cell number was counted via trypan blue exclusion on the indicated days. (C) Western blot analysis of BCL-xL protein levels after treatment with Fulvestrant plus Palbociclib on the indicated days. All images are representative fields or blots from at least two/three independent experiments.

3.2.8. ARV-825 extends growth delay and suppresses proliferative recovery in MCF-7 breast tumor cells treated with Fulvestrant + Palbociclib

Inhibitors of bromodomain-containing protein 4 (BRD4), particularly ARV-825, have demonstrated antitumor activity in multiple preclinical models, and have recently been considered as potential senolytics [143]. To investigate whether ARV-825 might act as a senolytic in combination with the senescence induced by the Fulvestrant + Palbociclib combination, cells were treated for 6 days with Fulvestrant (100 nM) and Palbociclib (1 μM), followed by ARV-825 (50 nM) for 96 h post-treatment. Temporal analysis of cell viability demonstrated that ARV-825, alone, moderately suppressed growth of the MCF-7 cells (**Figure 3.8.A**) without significant apoptosis (**Figure 3.8.B**); this is consistent with prior literature studies of the action of ARV-825 [144, 145], and with the degradation of BRD4 and the suppression of downstream c-Myc shown in **Figure 3.8.C**. Our laboratory as well as others has shown that c-Myc is upregulated in breast cancer and involved in breast cancer proliferation [144, 146, 147].

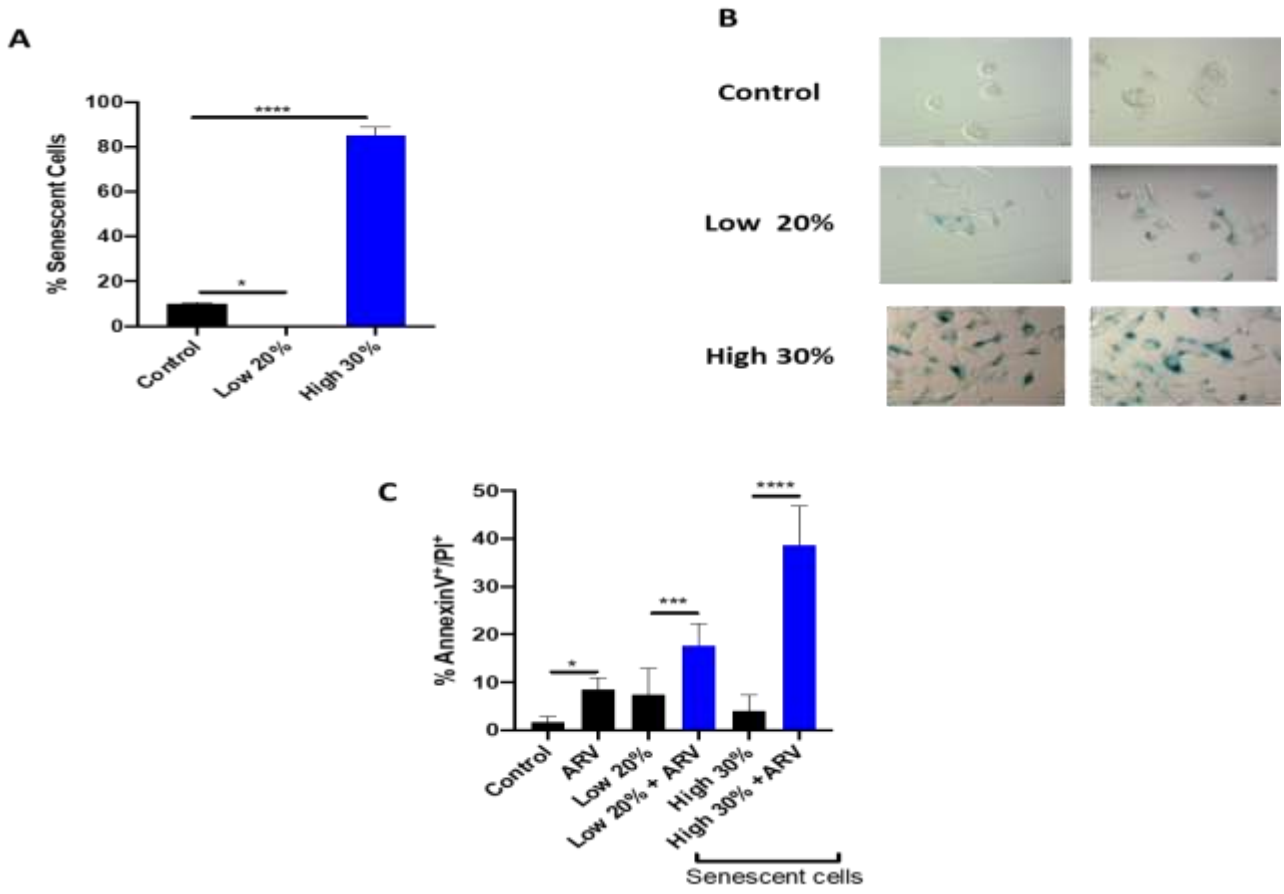
The most critical observation in this work is that ARV-825 treatment sequentially after the Fulvestrant + Palbociclib combination resulted in a prolonged growth arrest, significant apoptosis induction, with suppression of proliferative recovery (**Figure 3.8. A and B**). This finding is consistent with the pronounced suppression of both BRD4 and c-Myc for the combination of Fulvestrant + Palbociclib and ARV-825 in **Figure 3.8.C**.



3.8. ARV prolongs growth arrest induced by Fulvestrant in combination with Palbociclib in MCF-cells. Cells were treated with Fulvestrant (100nM) plus Palbociclib (1uM) for 6 days followe by ARV-25 (50Nm) addition for 4 days. (A) (#) Cell viability was monitored over a period of 14 days by trypan blue exclusion. (B)(#) Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit. (C) Western blotting for BRD4, c-Myc at day 4 of ARV treatment. *P ≤ 0.05, ****P ≤ 0.0001, ns (not significant) indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak's *post hoc* test. All images are representative fields, blots, or data from three independent experiments (n = 3).

3.2.9. The potential senolytic activity of ARV-825 in MCF-7 cells

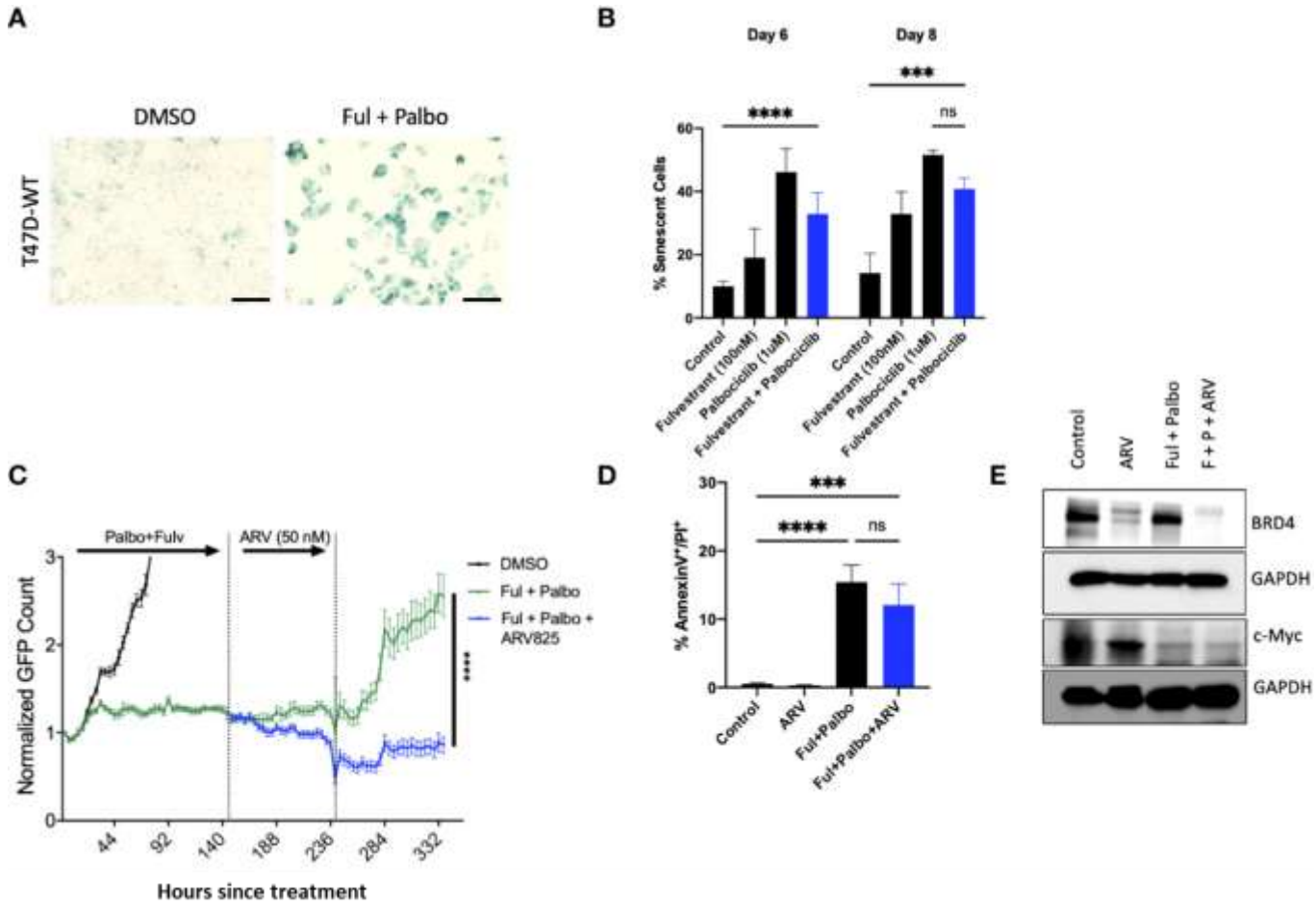
Although the combination treatment promotes substantial senescence in the MCF-7 cells (Figure 3.6), the entire cell population is not senescent, and consequently it was necessary to address whether the ARV-825 was functioning as a senolytic and that the senescent cell population might be particularly vulnerable to the ARV-825. To address this question, cells were sorted by flow cytometry to distinguish the SA-β-Gal highly positive and low positive populations (high 30% and low 20% SA-β-Gal positive) as well as the control. Flow cytometry as well as B gal staining were performed post-sorting for C12FDG staining to confirm the senescent population (**Figure 3.9.A and B**). **Figure 3.9.C** indicates that the senescent high cell population underwent significantly more apoptosis compared to the non-senescent cells after treatment with Fulvestrant/Palbociclib + ARV-825. These data indicate that the senescence induced by the combination of an anti-estrogen and CDK4/6 inhibitor increases susceptibility to ARV-825 induced apoptotic cell death.



3.9. ARV-825 showed a potential senolytic activity. Cells were treated with Fulvestrant + Palbociclib combination for 6 days prior to cell sorting. (A) (#) Flow cytometry was performed post-sort for C12FDG staining to confirm senescence induction. (B) Sorted cells were plated for 24-hours and stained with β-Gal staining to verify purity of the SA-β-Gal positive population (high 30% and low 20% SA-β-Gal positive). (C)(#) Sorted senescent cells were plated for 24-hours and treated with ARV-825 for 4 days followed by annexin V/PI apoptosis staining. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak's post hoc test. All images are representative fields, blots, or data from three independent experiments (n = 3).

3.2.10. Sensitization by ARV-825 in p53 mutant T-47D breast tumor cells treated with the Fulvestrant + Palbociclib combination

In order to evaluate whether ARV-825 would also be effective against p53 mutant ER+ breast tumors treated with the Fulvestrant + Palbociclib combination, we assessed the temporal response by real time, live cell imaging using IncuCyte S3 in T-47D breast tumor cells. Analogous to the outcomes in MCF-7 cells (**Figure 3.6**), we observed that the combination of Fulvestrant + Palbociclib induced senescence based on senescence associated β-galactosidase staining (**Figure 3.10. A**) that was followed by proliferative recovery (**Figure 3.10. C**); as in the studies with the p53 wild-type MCF-7 cells, the addition of ARV-825 resulted in prolonged growth arrest without recovery, at least over the ~ 13-day time course of this study (**Figure 3.10. C**). Quantifying the extent of senescence using C_{12}FDG staining indicated that approximately 40% of the population represented senescent cells at both day 6 and day 8 (**Figure 3.10. B**). Analysis of apoptosis indicated that there was no significant difference with the combination + ARV825, compared to the combination alone (**Figure 3.10. D**), despite some evidence of a decline in cell number in the temporal response study. Additionally, western blot analysis confirmed a reduction in the target protein, BRD4, by ARV-825 in both control and Fulvestrant + Palbociclib (**Figure 3.10. E**). Similarly, a profound suppression of downstream c-Myc by ARV-825 is evident in control cells, the combination alone and the combination + ARV825 (**Figure 3.10. E**).



3.10. ARV prolongs growth arrest induced by Fulvestrant in combination with Palbociclib in p53 mutant T-47D cells. T47D-WT cells that were treated with Palbociclib (1 µM) in combination with Fulvestrant (100 nM) for 6 days. (A) (Dr. Knudsen's Lab) Cells were fixed on Day 6, stained with x-gal staining solution, and imaged using brightfield microscope. All images were taken with the same magnification. (B)(#) Quantification of SA-Bgal using C12FDG at indicated timepoints. (C) (Dr. Knudsen's Lab) Live cell viability was monitored *via* IncuCyte over a period of 14 days and normalized to GFP count. (D) (#) Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit. (E) Western blotting for BRD4, c-Myc at day 4 of ARV treatment. Mean and SD were determined based on triplicates from 3 independent experiments. ***P ≤ 0.001, ****P ≤ 0.0001, ns (not significant) indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak's *post hoc* test.

3.2.11. ARV-825 effect in the organoids derived from MCF-7 and T47D

The impact of ARV-825 is further confirmed using 3-D culture organoids derived from MCF-7 and T47D cells (**Figure 3.11.A and B**). ARV-825 addition to the organoids treated with Fulvestrant plus Palbociclib for 6 days, results in a significant reduction in the surviving organoids as shown in **Figure 3.11.B**.

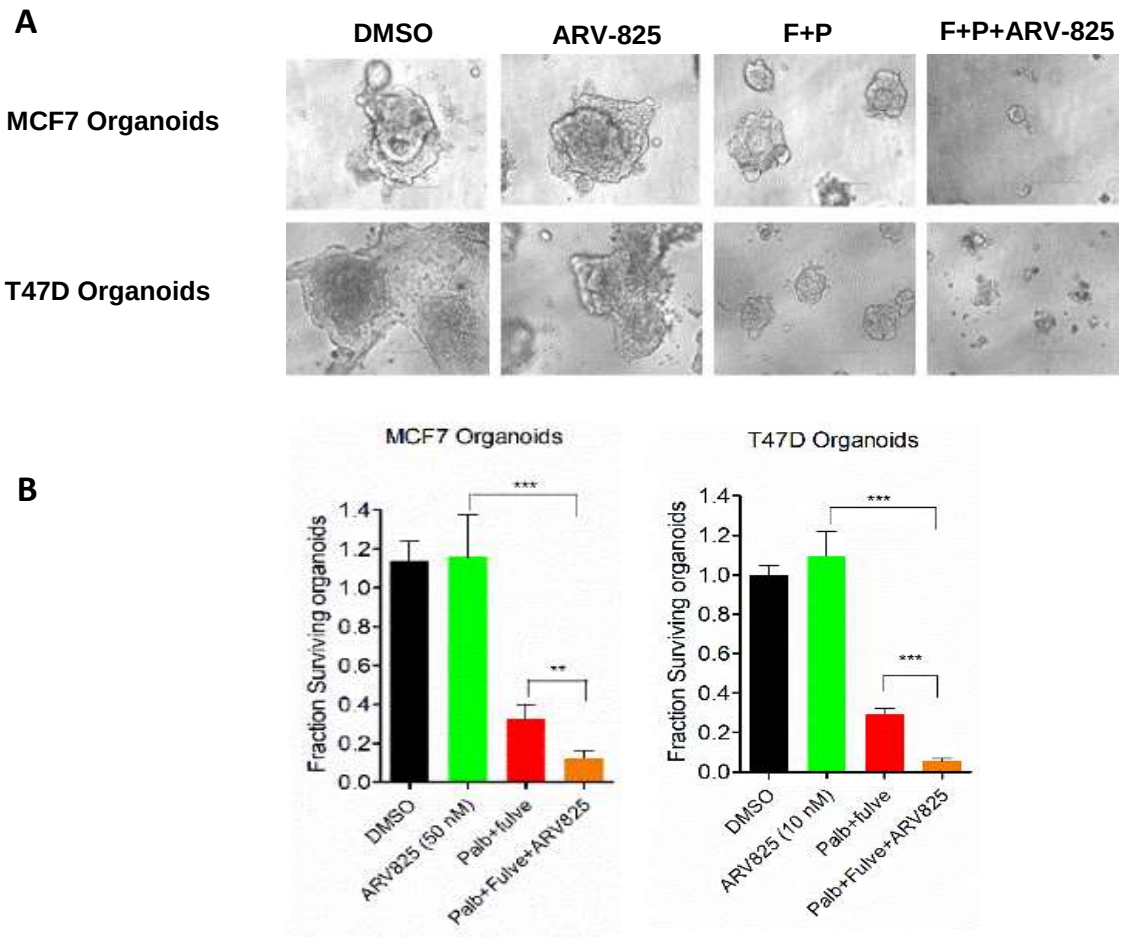


Figure 3.11. ARV-825 suppresses the growth of Fulvestrant + Palbociclib treated MCF-7 and T-47D organoids. A. Representative Images of organoids derived from MCF7 and T47D cells following the treatment with indicated drugs. Drug treatment was Palbociclib (1 μM) in combination with Fulvestrant (100 nM) for 6 days. Following 4-days of treatment ARV825 was removed and replaced with fresh media and the organoids were allowed to recover up to 13 days. The viability of organoids was determined by using CellTiter-Glo-3D assay. Graphs represent mean and SD from replicates (n=4). (***p<0.001, **p<0.01 as determined by student t-test). (Dr. Knudsen's Lab).

3.2.12. The growth arrest response of MCF-7 cells to ABBV-744

One major problem that challenges the utilization of BET inhibitors, including AZD5153, BMS 986158, and CPI-0610, is the serious side effects that reported recently [118]. These adverse effects range from mild symptoms, as diarrhea, nausea, and fatigue, to serious ones, such as thrombocytopenia, anemia, as well as neutropenia. Consequently, our main goal was to find a selective BET inhibitor with a better side effect profile. ABBV-744 is a selective BDII domain inhibitor that showed promising preclinical results in hematologic malignancies [148] as well as prostate cancer [149]. Therefore, we investigated the effect of ABBV-744 in various ER+ breast cancer models together with endocrine therapies. We initially assessed the response of MCF-7 cells to a range of concentrations of ABBV-744 (between 12.5-125 nM). ABBV-744 triggered moderate growth arrest at all concentrations tested, with minimal apoptosis **(Figure 3.12. A, and B)**. The lack of cell death was further confirmed by the LDH assay **(Figure 3.12.C**).

ABBV-744 is a potent inhibitor targeting the BDII domain of the BET family [149]. We evaluated the effects of different ABBV-744 concentrations on the BET family members, BRD2, BRD3, and BRD4. ABBV-744 did not significantly alter the levels of BRD2 or BRD3 (**Figure 3.12.D**); however, a significant downregulation in BRD4 levels was evident starting from 75nM and 100nM (**Figure 3.12.D**), indicating the potential selectivity of ABBV-744 towards BRD4 in ER+ breast cancer cells. Consequently, we chose 100nM as a suitable concentration for the subsequent studies.

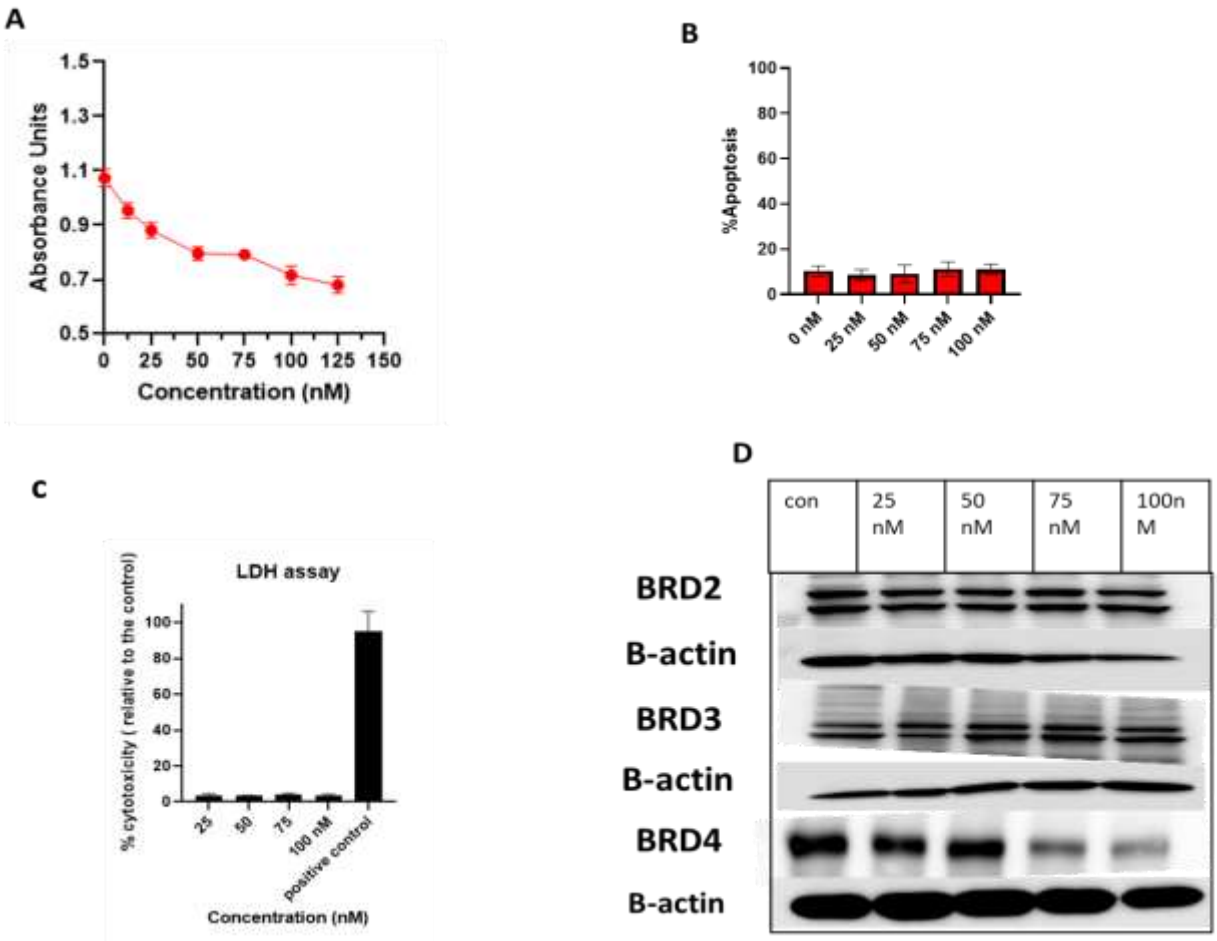


Figure 3.12. ABBV-744 induced a growth inhibitory response in MCF-7 cells without apoptosis induction. Cells were treated with ABBV-744 (12.5 nM, 25 nM, 50 nM, 75 nM, 100 nM and 125 nM) for 4 days and dose response curve was determined using MTS viability assay (A). Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit at day 4 of ABBV-744 indicated treatments (B). Cytotoxicity was measured using LDH assay kits at day 4 of ABBV-744 indicated treatments (**C).** Western blotting for BRD2, BRD3, and BRD4 at day 4 of ABBV-744 indicated treatments (D). All images are representative fields or blots from at least two/three independent experiments.

3.2.13. ABBV-744 extends the growth inhibitory response initiated by Fulvestrant plus Palbociclib in MCF-7 cells.

As shown in **Figure 3**, the combination of Fulvestrant plus Palbociclib drives cancer cells into a state of senescence, from which the cells escape between days 12-18 (**Figure 3.13.A**). **Figure 3.13.A** demonstrates that ABBV-744 (100nM) induces a modest but significant growth inhibitory response alone, consistent with the data in **Figure 3.12.A**; however, our main focus is to investigate the effect of ABBV-744 in combination with one of the standard cares of therapy, Fulvestrant plus Palbociclib, which will be more clinically relevant. **Figure 3.13.A** shows that ABBV-744 (100nM) addition at days 6 to 10 extends the growth inhibitory state mediated by Fulvestrant plus Palbociclib, although the cells do recover beginning at around day 16. This growth inhibition occurs without significant apoptosis induction, as indicated by Annexin V/PI based Flow cytometry (**Figure 3.13.B**). The extended growth inhibition induced by ABBV-744 does not reflect an increase in the extent of senescence that was initiated by Fulvestrant plus Palbociclib, as shown by flow cytometry quantification of C_{12}FDG fluorescence (**Figure 3.13.C**), highlighting that ABBV-744 mediates a specific non-senescent growth inhibition state.

Consistent with the data shown in **Figure 3.13.E**, ABBV-744 in combination with Fulvestrant plus Palbociclib causes a significant reduction in BRD4 levels. This is accompanied by downregulation of the BRD4 downstream effector, c-Myc, as shown by western blotting, either alone or in combination with Fulvestrant + Palbociclib (**Figure 3.13.D**). Interestingly, ABBV-744 in combination with Fulvestrant plus Palbociclib causes p53 accumulation together with its downstream effector, p21 (**Figure 3.13.D**), suggesting a potential role for the p53/p21 axis in growth inhibition mediated by ABBV-744.

To establish the apparent requirement for BRD4 suppression in sensitization, we tested a concentration of ABBV-744, 50nM, at which no reduction in BRD4 level was observed (**Figure 3.12.E**). **Figure 3.13.E and F** showed that ABBV-744 (50nM) neither prolonged the growth arrest mediated by Fulvestrant plus Palbociclib nor produced significant apoptosis, supporting the premise that BRD4 reduction is required for ABBV-744- mediated extended growth arrest.

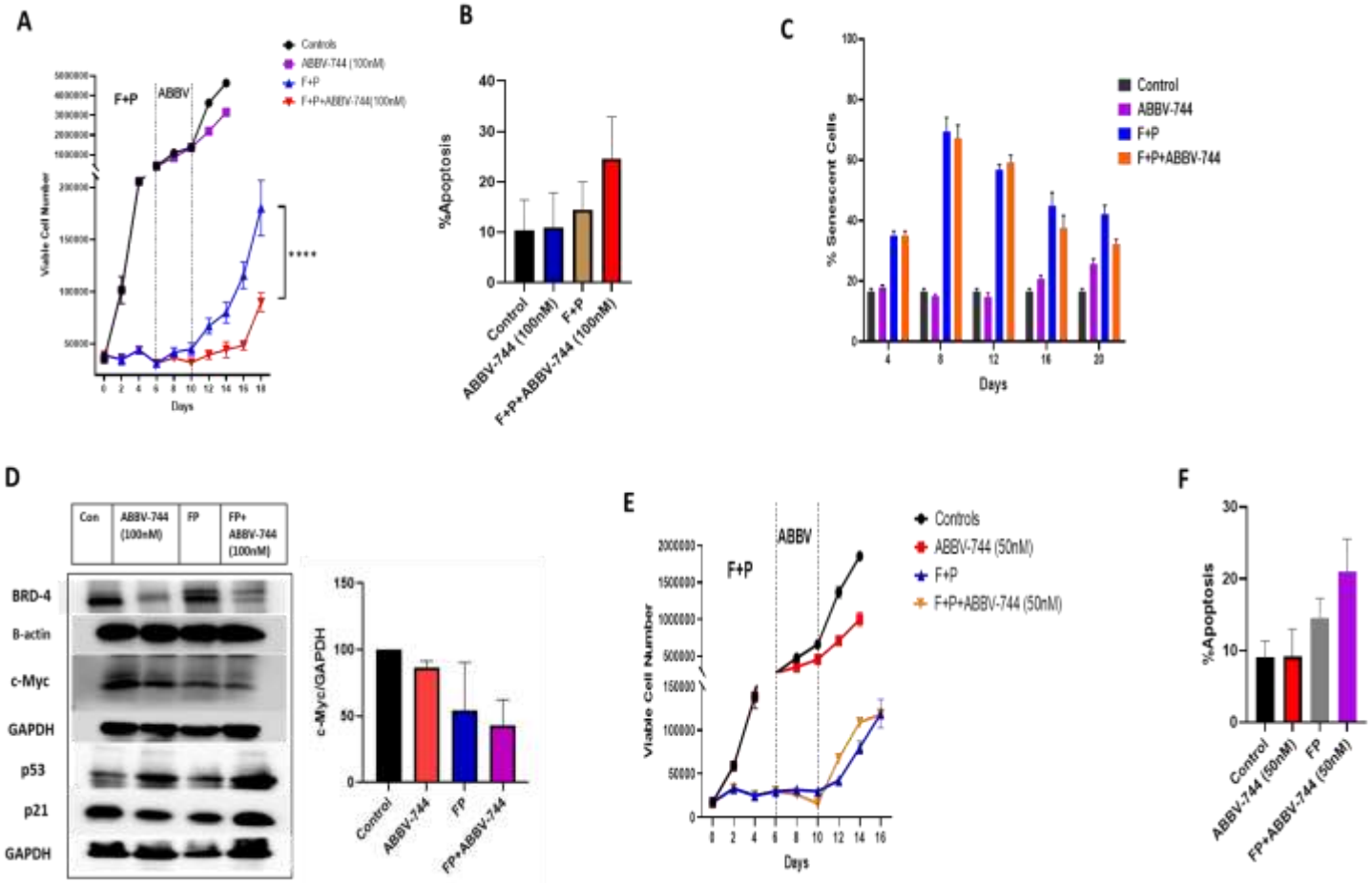


Figure 3.13. ABBV-744 extends the growth inhibitory response initiated by Fulvestrant plus Palbociclib in MCF-7 cell line. Cells were treated with Fulvestrant (100nM) plus Palbociclib (1uM) for 6 days then ABBV-744 (100nM) added starting from day 6 to 10 and cell viability was monitored over a period of 18 days by trypan blue exclusion (**A**). Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit (**B and F**). Quantification of SA-Bgal using C12FDG at indicated timepoints (**C**). Western blotting for BRD4, c-Myc, p53 and p21 at day 4 of ABBV-744 treatment (**D**). Cells were treated with Fulvestrant (100nM) plus Palbociclib (1uM) for 6 days then ABBV-744 (50 nM) added starting from day 6 to 10, cell viability was monitored over a period of 16 days by trypan blue exclusion (**E**). All images are representative fields or blots from at least two/three independent experiments. ****P $\leq$ 0.001 indicate statistical significance of each condition compared to Fulvestrant plus Palbociclib as determined using two-way ANOVA with Sidak's *post hoc* test.

3.2.14. ABBV-744 alone or in combination with Fulvestrant plus Palbociclib did not affect the proliferation of T47D cells.

We further investigated the effect of ABBV-744 on the response to Fulvestrant + Palbociclib in the p53 mutant T47D ER+ breast cancer cell line. **Figures 3.14.A, B and C** demonstrate that ABBV-744, in the dose range between 25-100nM, induces neither a growth inhibitory response nor apoptosis, as shown by MTS, proliferation assay, as well as by assessment of apoptosis. This is consistent with the observation that ABBV-744 resulted in a modest BRD4 reduction in the T47D cell line **(Figures 3.14.D)**.

As shown in **Figure 3.10**, Fulvestrant plus Palbociclib induced senescence in the T47D cell line, from which the cells recovered starting from days 12-14 (**Figures 3.14.E).** In contrast to its effect in MCF-7 cells, ABBV-744 (100nM) does not extend the growth inhibitory state that was initiated by Fulvestrant plus Palbociclib in the T47D cells **(Figures 3.14.E)**. Furthermore, the Annexin V/PI assay showed the lack of apoptosis either alone or in combination with Fulvestrant plus Palbociclib **(Figures 3.14.F).** Moreover, ABBV-744 alone or in combination with Fulvestrant plus Palbociclib showed a reduction in the levels of BRD-4, without affecting the levels of c-Myc (**Figures 3.14.G**), suggesting that the growth inhibitory effect mediated by ABBV-744 are likely to be p53 dependent. The next series of studies addressed the role of p53 in the actions of the ABBV-744.

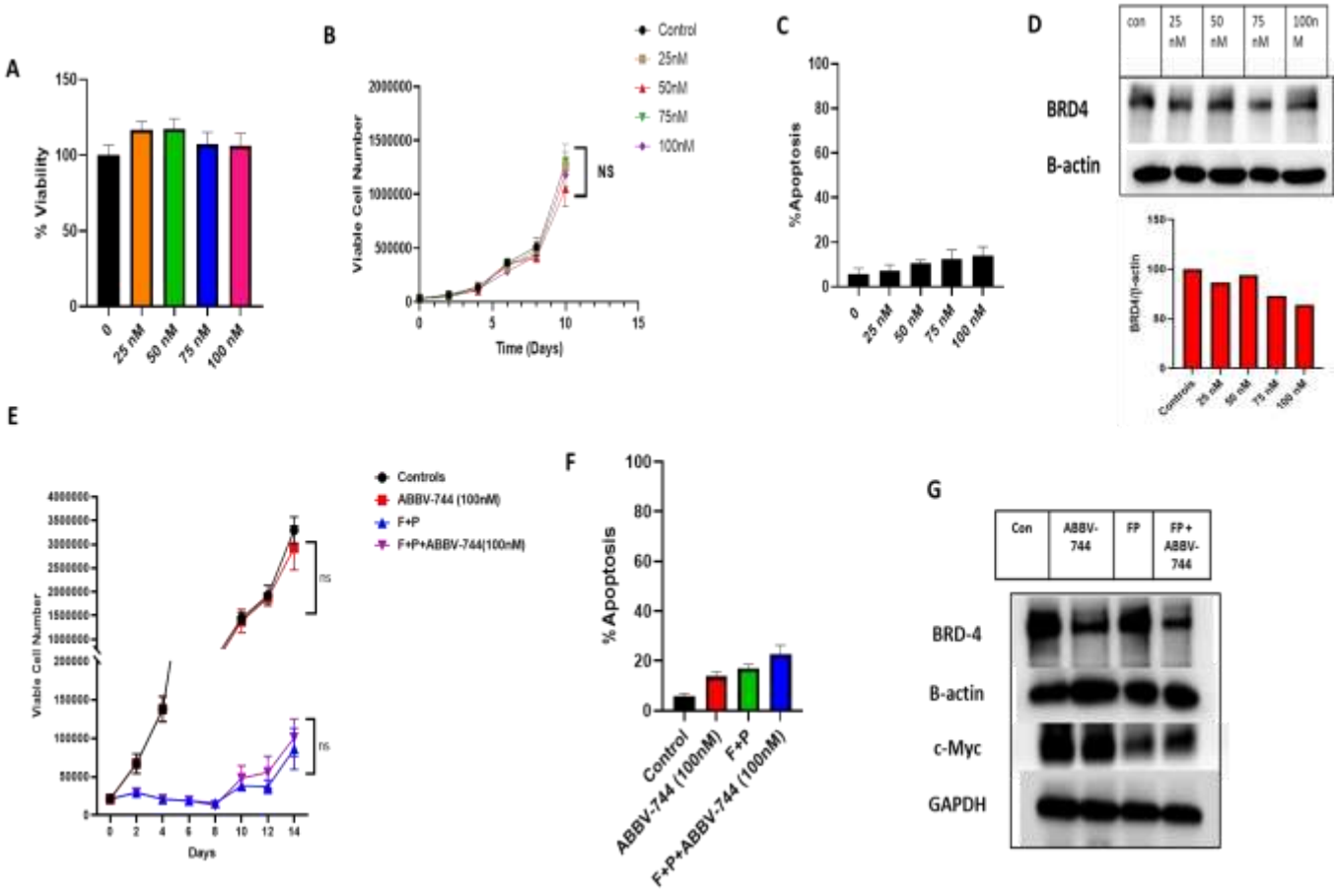


Figure 3.14. ABBV-744 does not extend the growth inhibitory response initiated by Fulvestrant plus Palbociclib in T47D cells. Cells treated with ABBV-744 (25 nM, 50 nM, 75 nM, and 100 nM) for 4 days. Percent cell viability was measured using MTS viability assay(**A**). Cell viability was monitored over a period of 10 days by trypan blue exclusion (**B**). Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit (**C).** Western blotting for BRD4 at day 4 of ABBV-744 (25 nM, 50 nM, 75 nM, and 100 nM) treatment **(D)**. Cells were treated with Fulvestrant (100nM) plus Palbociclib (1uM) for 6 days then ABBV-744 (100nM) added starting from day 6 to 10. Cell viability was monitored over a period of 14 days by trypan blue exclusion (**E**). Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit (**F**). Western blotting for BRD4, and c-Myc, at day 4 of ABBV-744 treatment (**G**). All images are representative fields or blots from at least two/three independent experiments. Ns (not significant) indicate statistical significance of each condition compared to control and Fulvestrant plus Palbociclib as determined using two-way ANOVA with Sidak's *post hoc* test.

3.2.15. The p53 dependent action of ABBV-744 in extending the growth inhibitory response initiated by Fulvestrant plus Palbociclib

To further confirm the role of p53 in mediating the effects of ABBV-744 in this experimental system, we combine ABBV-744 with pifithrin α in p53-WT MCF-7 cells. Pifithrin α is a small molecule which has been widely used as a specific inhibitor of p53 [150, 151] **(Figure 3.15.A)**. Pifithrin-α minimized the growth inhibitor effect mediated by ABBV-744 in MCF-7 cells. Furthermore, we performed studies in MCF-7 cells where p53 had been silenced using CRISPR/Cas9. We initially validated the knockout procedure using western blotting, which showed the absence of p53 bands even after the treatment with different concentrations of doxorubicin [152] **(Figure 3.15.B).**

Fulvestrant plus Palbociclib causes growth arrest in MCF-7 p53 -/-, with the cells beginning to recover between days 12- 18 **(Figure 3.15. C)**. Consistent with the data generated in the T47D cell line, ABBV-744 addition to the cells treated with Fulvestrant plus Palbociclib did not affect p53 -/- MCF-7 growth, in contrast to MCF-7 WT cells, in which ABBV-744 extend the growth arrest mediated by Fulvestrant plus Palbociclib (**Figure 3.15. C)**, Furthermore, ABBV-744 either alone or in combination with Fulvestrant plus Palbociclib showed BRD4 reduction without affecting c-Myc levels (**Figure 3.15. D)**, confirming the apparent p53 dependency for the ABBV-744 mediated effect.

These results together with the data in **Figure 3.13** showed that ABBV-744 mediated growth inhibition to the cells treated with Fulvestrant plus Palbociclib is dependent on BRD4, c-Myc reduction as well as p53 action. Based on these results, we next evaluated whether there was a physical interaction between p53 and BRD4 in MCF-7 cells. This was confirmed by a

Immunoprecipitation assay (**Figure 3.15.D**), in which BRD4 immunoprecipitation resulted in p53 accumulation, in the same time, p53 Immunoprecipitation, resulted in BRD4 accumulation.

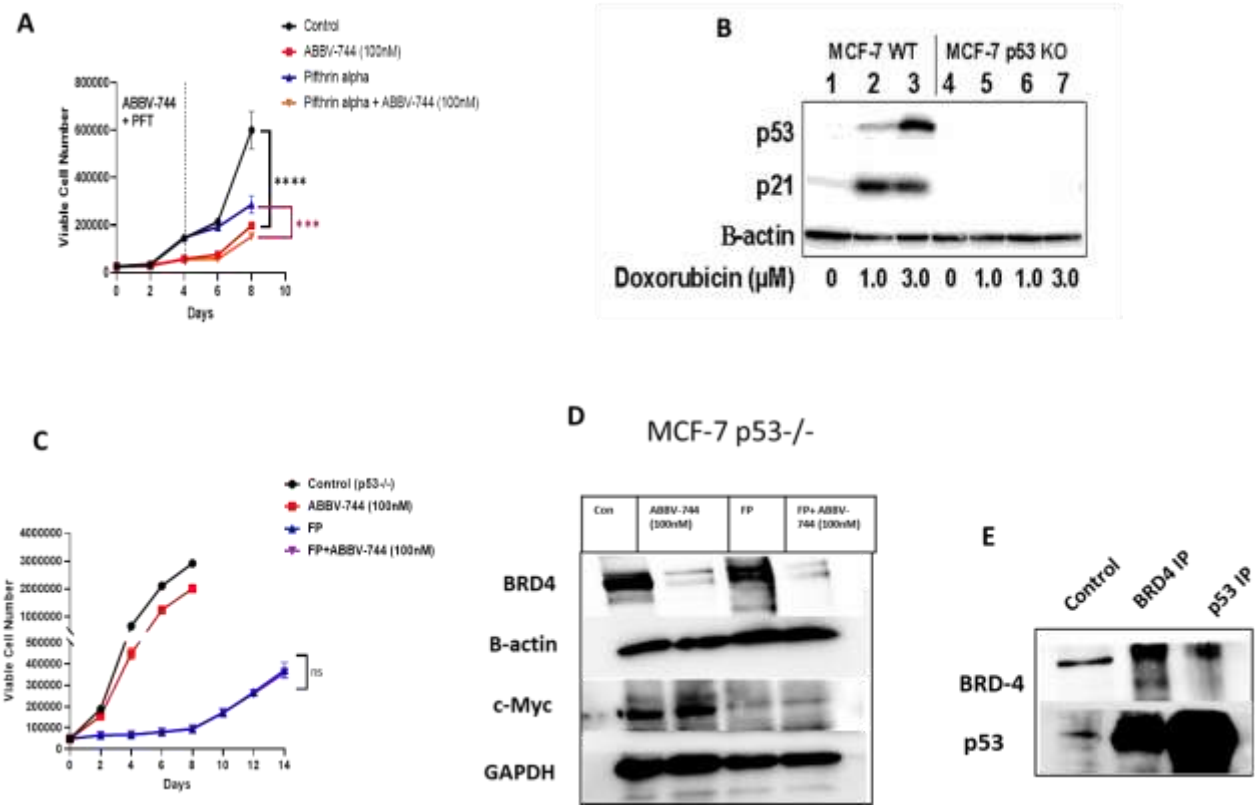


Figure 3.15. ABBV-744 activity in MCF-7 p53-/- cells. MCF-7 WT cells treated with ABBV-744 (100nM), and ABBV-744 (100nM) in combination with pifithrin α for 4 days and cells viability was monitored over 8 days using trypan blue (**A**). Western blots for p53, p21 and B-actin after 24h of doxorubicin treatment (Dr. Schoenlein's lab) (**B**). MCF-7 p53-/- were treated with Fulvestrant (100nM) plus Palbociclib (1uM) for 6 days then ABBV-744 (100nM) added starting from day 6 to 10 and cell viability was monitored over a period of 18 days by trypan blue exclusion (**C**). Western blots for BRD4 and c-Myc at day 4 of ABBV-744 treatment (**D**). Using untreated MCF-7 WT cells, Immunoprecipitation for BRD4 and p53 using BRD-4 antibodies (**E**). All images are representative fields or blots from at least two/three independent experiments. ***P ≤ 0.001, ****P ≤ 0.001, ns (not significant) indicate statistical significance of each condition compared to control, Pifithrin alpha, and Fulvestrant plus Palbociclib as determined using two-way ANOVA with Sidak's *post hoc* test.

3.4. Discussion

Disease recurrence, both local and distal, is an ongoing issue contributing to the majority of hormone receptor-positive breast cancer deaths, and is observed in many other types of cancers, such as triple-negative breast cancer, lung, and prostate cancer. Often this recurrence can be associated with a therapy-induced residual dormant tumor cell population that can escape and often become more aggressive in nature [153-155]. While therapy-induced senescence has been studied for decades, successful utilization of senolytics in cancer treatment has not yet been implemented. Despite this, several senolytic agents have been considered and studied to modulate and eliminate senescent tumor cells.

Another potential route for modulation of drug sensitivity in cancer is autophagy inhibition [156, 157]. Estrogen receptor-targeted therapies are generally the first-line treatment for hormone receptor-positive breast cancer and autophagy induction has been shown in response to these agents, where autophagy may played a cytoprotective role [158]. In fact, this form of autophagy has been shown to lead to the development of resistance to anti-estrogen therapies [159-161]. In cases where autophagy is cytoprotective, pharmacological inhibition of autophagy may be utilized to enhance the tumor cell sensitivity to treatment. In addition to cytoprotective autophagy, we have identified a non-protective form of autophagy, which apparently plays no distinct role in promoting or suppressing the growth or sensitivity of the tumor cells in response to therapy [48, 157, 162]. Clinical trials are currently underway evaluating HCQ as a pre-treatment with the combination of letrozole + Palbociclib, based on preclinical studies showing efficacy in autophagy inhibition as a pretreatment with this combination [12].

The current work evaluated the potential of utilizing autophagy inhibition to sensitize ER positive MCF-7 breast tumor cells to the combination of Fulvestrant + Palbociclib. This treatment promotes significant growth arrest and both autophagy and senescence induction after 6 days of treatment with Fulvestrant and Palbociclib. Slight sensitization was evident with the addition of pharmacological autophagy inhibitors as well as with genetic knockdown; however, these approaches did not improve tumor cell responsiveness to the combination treatment, leading to the conclusion that the autophagy was non-protective and that autophagy inhibition is unlikely to become a clinically useful therapeutic strategy [157, 163]. This does not rule out the possibility that the autophagy induced by aromatase inhibitors in combination with cdk4/6 inhibitors could be cytoprotective and amenable to autophagy inhibition in the clinic.

Our studies further examined the incorporation of a BET degrader, ARV-825, into the combination treatment. We initially screened a variety of agents from different drug classes that had been reported to have senolytic activity including ABT-199, as well as ABT-263, and found that this BET inhibitor was the most promising agent. BET inhibition has demonstrated efficacy in many clinical trials consisting of both hematological malignancies as well as solid tumors [118]. The BET degrader ARV-825 has been used in pre-clinical studies with different cancer types, and we hypothesized that ARV-825 could potentially improve ER+ breast cancer tumor response following Fulvestrant + Palbociclib treatment [154, 155, 157, 158]. ARV-825 suppressed tumor growth for both the ER positive p53 WT MCF-7 cells and the ER positive p53 mutant T-47D cells, significantly delaying proliferative recovery. We also observed significant induction of apoptosis in the MCF-7 cells treated with Fulvestrant + Palbociclib followed by ARV-825, but not in T47D cells, possibly due to p53 status in these cells. The senescent MCF-7 cell population appears to be more susceptible to ARV-825 induced apoptosis, although a low

degree of apoptosis is also observed in non-senescent cells exposed to the ARV-825. The impact of ARV-825 on tumor cell sensitivity to Fulvestrant + Palbociclib was further confirmed using organoids derived from MCF-7 and T47D cells.

The observed growth arrest is consistent with the degradation of BRD4 and the suppression of downstream c-Myc as well as with previous studies by our laboratory and others demonstrating c-Myc to be upregulated in ER+ breast cancer and involved in breast cancer proliferation [144, 146, 147]. These findings are supported by an analysis of three patient database sets, demonstrating that high expression of BRD4 that can be observed across multiple subtypes of breast cancer is correlated with overall lower recurrence-free survival when compared to patients with low BRD4 expression levels [164].

Another BET inhibitor that being investigated in clinical trials for relapsed/refractory Acute Myeloid Leukemia (AML) (**NCT03360006**) and which has shown promising results in prostate cancer models [149] is ABBV-744. In the current work, ABBV-744 extended the growth inhibitory response mediated by Fulvestrant plus Palbociclib at the concentration where BRD4 was downgraded without either increasing the magnitude of senescence or apoptosis in the MCF-7 cell line. ABBV-744 inhibits BRD4, which is needed for ABBV-744 mediated growth inhibition in combination with Fulvestrant plus Palbociclib, together with c-Myc down regulation, while ABBV-744 has minimal impact on BRD2 and 3. Interestingly, ABBV-744 appeared to demonstrate a p53-dependency as validated by studies in the p53 mutant T-47D cell line, pifithrin α, as well as by CRISPR-mediated KO of p53 in MCF-7 cells. This p53 dependency is consistent with several publications [165-168] that have investigated the possible relationship between p53 and BET proteins, especially BRD4 [169]. Recently, Wu et al. [165] have shown that BRD4 interacts with p53, and that this interaction is modulated by casein

kinase II (CK2), which mediates the phosphorylation of a conserved acidic region in Brd4 protein ,dictating the chromatin binding of Brd4 as well as recuriting p53 to regulated promoters [165]. Based on these data, further investigation is needed to understand the relation between BRD4, c-Myc as well as p53, in order to validate the hypothesis that ABBV-744 may be disrupting the connection between p53 and BRD4, affecting c-Myc.

Chapter 4: BRD4 targeting as a possible strategy in combination with Tamoxifen in ER-positive breast cancer

4.1. Introduction

Another major class of endocrine therapies involves the utilization of selective estrogen receptor modulators (SERMS), primarily Tamoxifen (TAM). TAM is one of the oldest and most frequently utilized SERMs, which competes with estrogen at the receptor site, blocking the estrogen promotional role in breast cancer [9]. TAM is typically prescribed to treat pre-menopausal women with early-stages of hormone receptor-positive breast cancer after surgery to reduce disease recurrence [8]. However, TAM has now been largely replaced by aromatase inhibitors such as Letrazole, in combination with CDK4/6 inhibitors including Palbociclib [6]. Recently, efforts to increase the effectiveness of TAM in treating ER+ breast cancer have emerged [99, 170].

4.1.1. Autophagy in response to Tamoxifen in ER+ breast cancer

A large number of studies in the literature have investigated the relationship between the development of Tamoxifen (TAM)-based therapy resistance and autophagy, based on the evidence that Tamoxifen induces a cytoprotective form of autophagy. For instance, Qadir et al. [159] investigated the role of TAM-induced autophagy in different breast cancer cell lines, including the ER+ breast cancer MCF-7 and T-47D cell lines. Autophagy induction in response to TAM in different breast cancer cell lines was confirmed by the accumulation of GFP-LC3 puncta, monodansylcadaverine (MDC) staining as well as with the lysosomal marker, Lysotracker. Importantly, autophagy inhibition via siRNA targeting of Atg7, Atg5, and Atg8

(Beclin-1) combined with TAM caused a dramatic reduction in MCF-7 cell viability compared to that of control cells treated with TAM + non-targeting (scrambled) siRNA. Similar outcomes were reported in studies utilizing the T47D cells [159]. These experimental outcomes indicated that autophagy inhibition can sensitize antiestrogen-sensitive and resistant ER (+) breast cancer cells to TAM-induced cytotoxic effects, specifically mitochondrial depolarization followed by caspase-9 activation and apoptosis via the intrinsic pathway [159]. A cytoprotective role for autophagy in the actual development of antiestrogen resistance (acquired resistance) was also demonstrated utilizing MCF-7 cells and an antiestrogen resistant MCF-7 subline that was selected with a stepwise selection protocol utilizing 4-hydroxytamoxifen [158]. Recently, studies have suggested that the utilization of BET inhibitors cause growth arrest via induction of a cytoprotective form of autophagy [171].

4.2. Overarching hypotheses

There are emerging efforts [99, 170] evaluating the possibility of increasing sensitivity to TAM by the utilization of BET inhibitors in combination with TAM. Therefore, we investigated the possible utilization of ARV-825 or ABBV-744 in combination with Tamoxifen, also addressing the possible involvement of autophagy.

4.3. Results

4.3.1. ABBV-744 did not extend the growth arrest mediated by Tamoxifen in MCF-7 cell line

Lee et al. [172] showed that TAM, at 10μM, induced senescence in the MCF-7 cell line. However, treatment with a more clinically relevant concentration of TAM, 5μM for 4 days, did not drive MCF-7 cells into a significant senescence, as shown by β-galactosidase staining (**Figure 4.1.A**). The absence of senescence was further confirmed by flow cytometry quantification of C_{12}FDG fluorescence (**Figure 4.1.B).** Instead, TAM treatment for four days resulted in a non-senescent transient growth suppression with ~ 20% apoptosis as compared to ~ 10% apoptosis in the controls; the cells appeared to recover full proliferative capacity between days 12 and 14 (**Figures 4.1.C and D)**. As consistent with the literature [8, 159, 173], TAM induces a significant autophagic flux as confirmed by acridine orange (AO) staining (**Figures 4.1.F).** The autophagy induction is further confirmed with autophagy markers p62 and LC3 I/II by western blotting assay (**Figures 4.1.E)**.

In contrast to the results with Fulvestrant plus Palbociclib**, Figures 4.1.C and D** shows that ABBV-744 neither induces growth inhibition nor apoptosis following Tamoxifen treatment for 4 days in MCF-7 cells. Furthermore, ABBV-744 in combination with TAM produced BRD4 downregulation, and p53 accumulation, without affecting c-Myc levels **(Figure 4.1. E).** Interestingly, ABBV-744 in combination with TAM showed no change in p21 level. Moreover, ABBV-744 alone triggers a marked autophagy; however, in combination with TAM, ABBV-744 resulted in a drastic autophagic flux in MCF-7 cells as shown by AO staining, p62 and LC3 I/II levels (**Figure 4.1.E and F**).

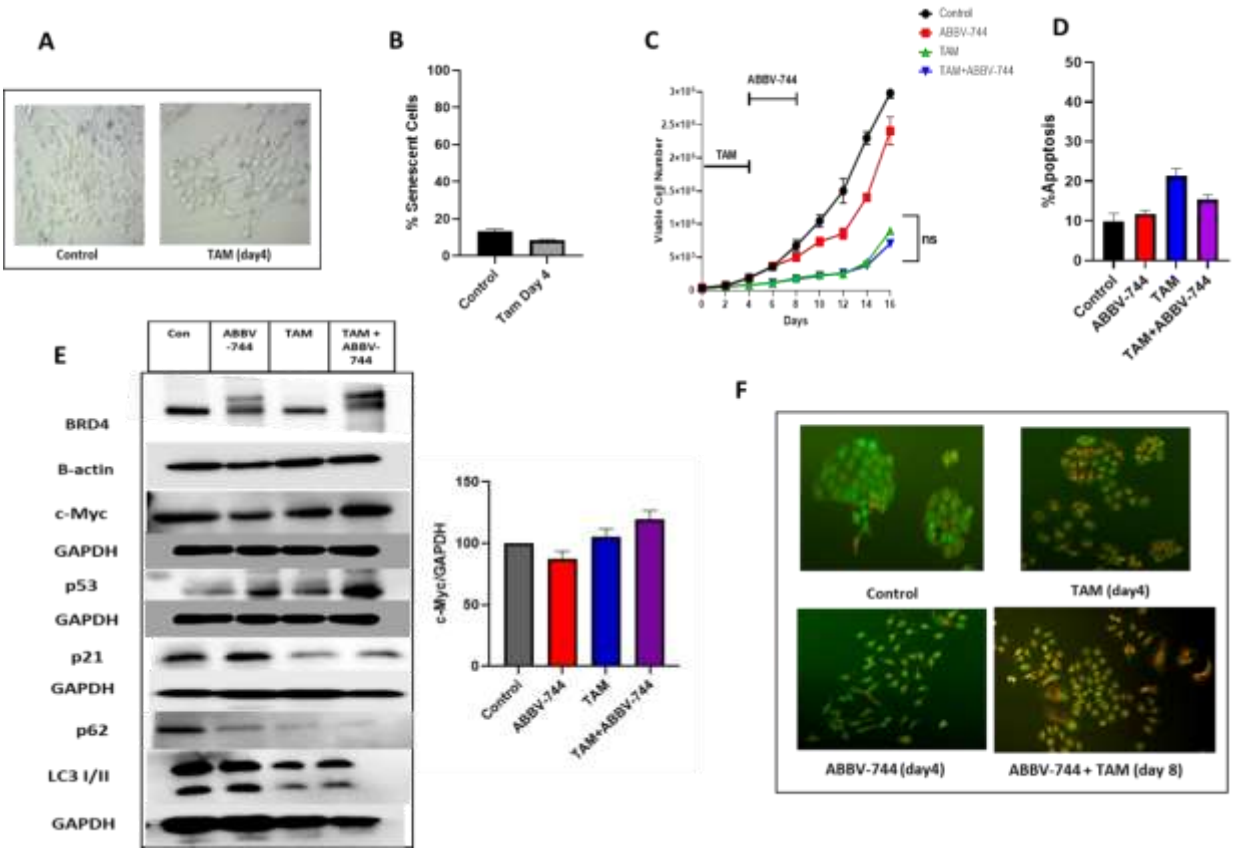


Figure 4.1. ABBV-744 did not suppress the recovery of TAM-treated cells. MCF-7 cells treated by tamoxifen for 4 days and Cells were fixed on Day 4, stained with x-gal staining solution and imaged using bright field microscope. All images were taken with the same magnification (A). Quantification of SA-B gal using C12FDG at indicated timepoints (**B**). Cells treated with Tamoxifen (5mM) for 4 days then ABBV-744(100nM) add starting from day 4 to day 8. Cell viability was monitored over a period of 16 days by trypan blue exclusion (**C**). Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit (**D).** Western blotting for BRD4, c-Myc, p53, p21, p62 and LC3 I/II at day 4 of ABBV-744 treatment (**E**)**.** Cells were stained with acridine orange on the indicated days and imaged using a fluorescent microscope. All images were taken at the same magnification (scale bar= 200 µm, n=3) (**F**). All images are representative fields or blots from at least two/three independent experiments. Ns (non-significant) indicates statistical significance of each condition compared to F+P as determined using two-way ANOVA with Sidak's *post hoc* test.

4.3.2. ARV-825 delayed the recovery of Tamoxifen-treated MCF-7 cells

After establishing the nature of the interaction of ARV-825 with Fulvestrant + Palbociclib [174], we investigated the possibility of combining ARV-825 (50 nM) with Tamoxifen (5 μM) in MCF-7 cells. ARV-825 alone induced a transient growth arrest largely similar to that of the Tamoxifen, as well as a similar degree of apoptosis (**Figures 4.2.A and B)** with recovery in roughly the same time frame as for TAM. However, the ARV treatment did not show either senescence or autophagy induction **(Figures 4.2.C, D, and E)**. The addition of ARV-825 to cells treated with Tamoxifen caused a reduction in cells number with a marked increase in the extent of apoptosis (**Figure 4.2.A and B**), as well as interference with the proliferative recovery that is observed with either agent alone (**Figure 4.2.A).** These observations along with the lack of senescence induced by the Tamoxifen indicate that in the context of the current studies, ARV-825 is not acting solely as a senolytic agent. We also observe a dramatic increase in autophagy levels with ARV-825 in combination with TAM at day 8 **(Figure 4.2. D, and E)**.

Importantly, ARV-825 either alone or in combination with TAM produced a significant reduction in BRD4 levels as well as in the levels of BET family members BRD2 and BRD3 (**Figure 4.2.D**). ARV-825 also produced a significant reduction in the BRD4 downstream effector, c-Myc **(Figure 4.2.D)**.

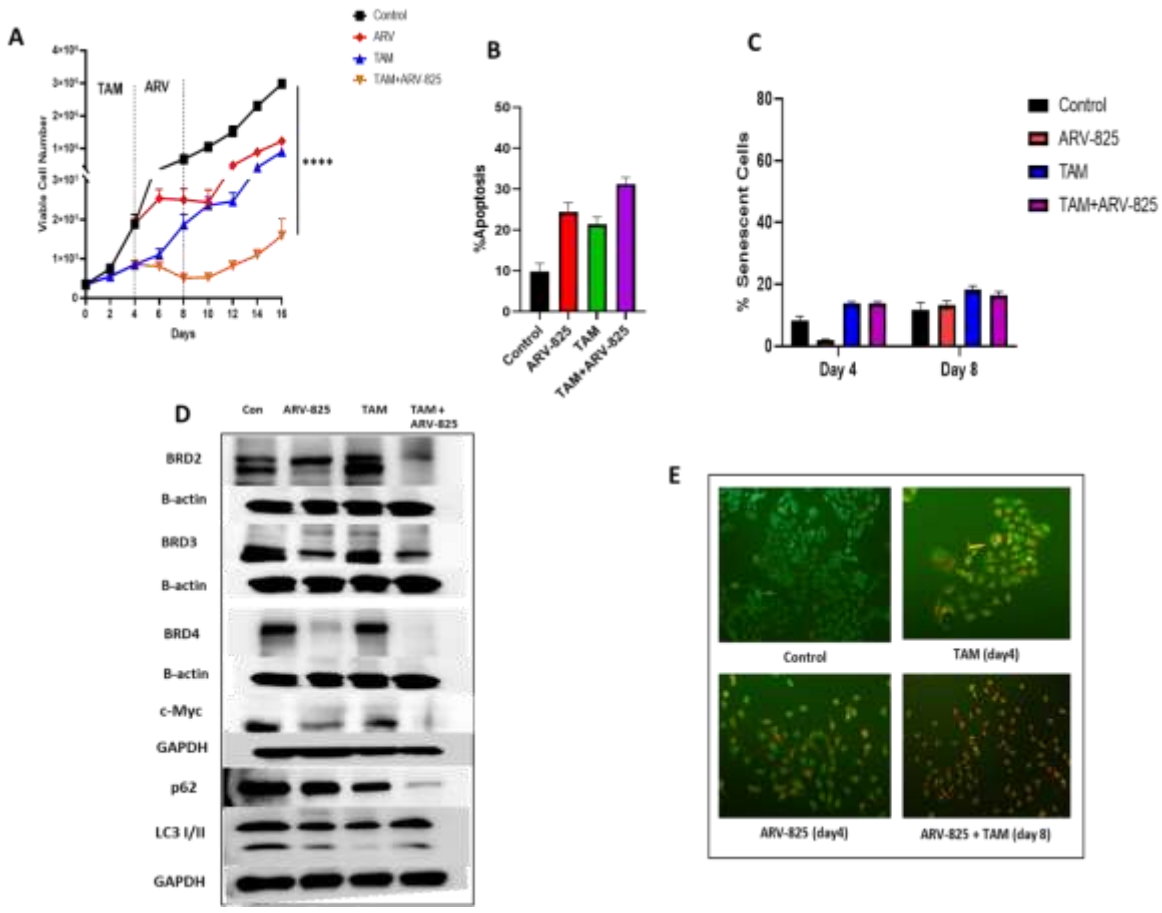


Figure 4.2. ARV-825 suppress the proliferative recovery of TAM-treated MCF-7 cell line. Cells treated with Tamoxifen (5mM) for 4 days then ARV-825(50nM) added starting from day 4 to day 8. Cell viability was monitored over a period of 16 days by trypan blue exclusion (**A**). Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit (**B).** Percent of SA-B gal is quantified using C12FDG at indicated time points (**C**). Western blotting for BRD4, c-Myc, p62, and LC3 I/II, at day 4 of ARV-825 treatment (**D**). Cells were stained with acridine orange on the indicated days and imaged using a fluorescent microscope. All images were taken at the same magnification (scale bar= 200 µm, n=3) (**E**). All images are representative fields or blots from at least two/three independent experiments. ****P ≤ 0.001 indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak's *post hoc* test.

4.3.3. ARV-825 also enhances the response to Tamoxifen in the p53 knockout MCF-7 breast tumor cell line

In our previous experiments with Fulvestrant + Palbociclib, ARV-825 enhanced the response in both the p53 wild type MCF-7 cells and in the p53 mutant T47D cells (**Figure 3.10**). Consequently, we investigated the effect of ARV-825 on the response to TAM in the p53 -/- MCF-7 ER+ breast cancer cell line. As was the case with MCF-7 WT cells, **Figure 4.3.A, B, and C** showed that TAM treatment resulted in a transient growth arrest followed by proliferative recovery without either significant apoptosis or induction of senescence in MCF-7 p53-/- cells. Furthermore, TAM induced a significant autophagy as shown by AO staining as well as by the autophagy markers p62 and LC3 I/II (**Figure 4.3.D and E**).

Again, similar to what was observed in MCF-7 WT cells, ARV-825 alone induce a transient growth arrest without senescence or marked apoptosis in MCF-7 p53-/- cells (**Figure 4.3.A, B, and C)**. However, ARV-825 addition to the cells treated with TAM suppressed cells proliferative recovery without either apoptosis or senescence (**Figure 4.3.A, B, and C)**. Interestingly, ARV-825 alone induce a marked autophagy in MCF-7 p53-/- cells; moreover, TAM in combination with ARV-825 caused a drastic increase in the autophagic flux (**Figure 4.3.D and E)**. Additionally, ARV-825 either alone or in combination with TAM showed a reduction in both BRD4 as well as c-Myc levels (**Figure 4.3.D)**.

Collectively, TAM in combination with ARV-825, unlike ABBV-744, resulted in suppression of the proliferative recovery of TAM-treated cells, together with a reduction in c-Myc level.

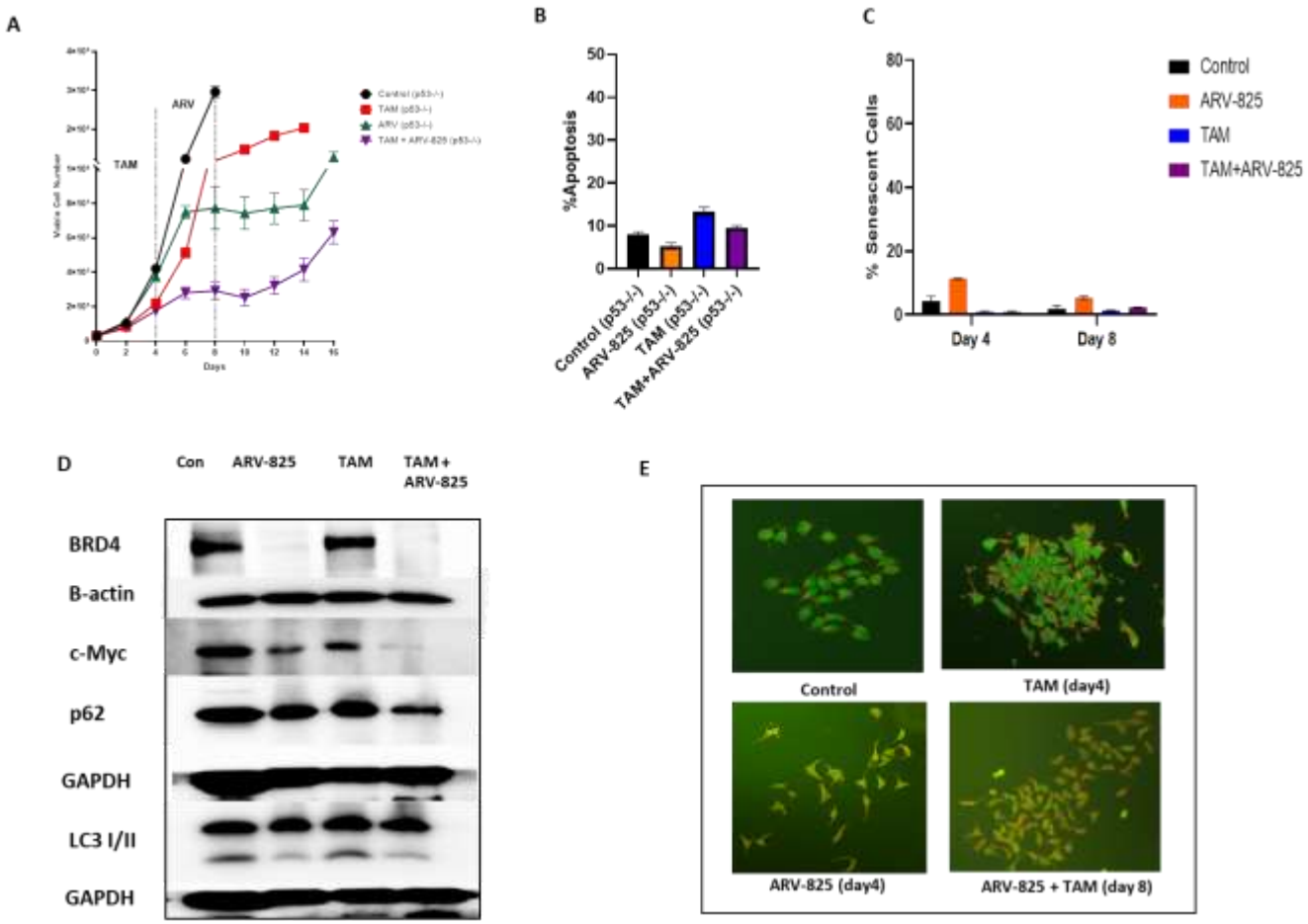


Figure 4.3. ARV-825 induces a growth suppression and autophagy in combination with TAM in p53-/- cells. MCF-7 p53-/- cells treated with Tamoxifen (5mM) for 4 days then ARV-825(50nM) added starting from day 4 to day 8. Viable cell number was monitored over a period of 16 days by trypan blue exclusion (**A**). Apoptosis was evaluated by flow cytometry using an APC Annexin V Apoptosis Detection Kit (**B).** Percent of SA-B gal is quantified using C12FDG at indicated time points (**C**). Western blotting for BRD4, c-Myc, p62, and LC3 I/II, at day 4 of ARV-825 treatment (**D**). Cells were stained with acridine orange on the indicated days and imaged using a fluorescent microscope. All images were taken at the same magnification (scale bar= 200 µm, n=3) (**E**). All images are representative fields or blots from at least two/three independent experiments. ****P ≤ 0.001 and ns (non-significant) indicate statistical significance of each condition compared to control as determined using two-way ANOVA with Sidak's *post hoc* test.

4.4. Discussion

Another class of endocrine therapies involves the utilization of selective estrogen receptor modulators (SERMS) including Tamoxifen (TAM). TAM is one of the oldest and most frequently utilized SERMs, which competes with estrogen at the receptor site, blocking the estrogen promotional role in breast cancer [9]. TAM is typically prescribed to treat pre-menopausal women with early-stages of hormone receptor-positive breast cancer after surgery to reduce disease recurrence[8].

Several functional forms of autophagy have been identified in response to different chemotherapeutic modalities [8, 128, 175-180], specifically cytoprotective, non-cytoprotective, cytostatic and cytotoxic autophagy [43, 179]. In our previous publication [174], we showed the non-protective role of autophagy-induced by Fulvestrant plus Palbociclib. Autophagy induction in response to Tamoxifen has been shown quite convincingly to be cytoprotective in many studies together with autophagy was the main pathway which promotes resistance to Tamoxifen [8, 158, 159, 181, 182]. In cases where autophagy is cytoprotective, pharmacological inhibition of autophagy may be utilized to enhance the tumor cell sensitivity to treatment; however, autophagy targeting is not clinically successful due to the difficulty of measuring autophagy inhibition in patients together with the absence of selective agents.

Initially, we tested ABBV-744 in combination with TAM in MCF-7 cells and observed that ABBV-744 did not induce either apoptosis or growth arrest. Furthermore, there is a reduction in BRD4 with no change in c-Myc levels when ABBV-744 is combined with TAM. The question why ABBV-744 did not work in combination with Tamoxifen has two possible answers and both of them need further investigations. Initially, several studies investigated the relation between TAM and p53 and that p53 is frequently related to the reduced response to TAM or the development of TAM resistance [183-190]. Importantly, Bailey et al. [191] have

been shown that TAM promotes p53 antagonism; furthermore, Guillot et al. [192] showed that TAM treatment causing alterations in p53 transcription. Lynnette et al. also showed that TAM–mediated effects are related to p53 status [193]. These findings are consistent with our results, in which ABBV-744 p53-dependant action is antagonized by TAM anti-p53 effects. Another possible explanation is autophagy induction, which may have a role in the absence of an ABBV-744 effect when combined with TAM, but again both answers need to be further investigated in the future studies.

On the other hand, ARV-825 showed promising results either alone or markedly with TAM. ARV-825 suppresses the TAM-treated population together with inducing a significant degree of apoptosis in MCF-7 WT but not in p53 KO cells, which may be due to the absence of p53 and its effect on the apoptotic process [194]. Mechanistically, and as was the case with Fulvestrant plus Palbociclib [174], ARV-825 demonstrated a reduction in BRD 2, 3 and BRD4 (most significant) expression levels together with suppressing c-Myc expression. Therefore, ARV-825 anti-proliferative activity may be mediated through the induction of BRD4 inhibition/degradation [169], leading to the suppression of its BRD4 downstream effector, c-Myc, a mechanism which is also supported by other labs [145, 195, 196]; however, more investigations are needed to confirm this pathway using BRD4/c-Myc overexpressing cells. Notably, the current study showed that ARV-825 is not only affecting the senescent population but also the non-senescent cells, as TAM did not induce senescence at a clinically relevant concentration (5uM).

As the case with ABBV-744, ARV-825 showed a drastic increase in autophagic flux when combined with TAM in MCF-7 WT and MCF-7 P53-/- cells without senescence, emphasizing that senescence does not always occur in parallel with autophagy [56, 197, 198].

This may highlight the difference between ARV-825 and ABBV-744 when combined with TAM, as ARV-825 caused c-Myc suppression, which may be the main reason for the growth inhibition mediated by ARV-825 alone or in combination with TAM,; this is not the case with ABBV-744, as no reduction in c-Myc was observed when ABBV-744 combined with TAM, that may be attributed to either the anti-p53 effect of TAM and the possible connection between BRD4, c-Myc and p53 or due to autophagy induction.

Chapter 5: Future Directions

In trying to increase the effectiveness of the standard drugs that are being utilized in the treatment of ER+ breast cancer, this work describes autophagy inhibition as well as targeting

epigenetic dysregulation, together with its association with senescence, as a potential strategy to increase the efficacy of the standard care of therapy for estrogen positive breast cancer. Initially, we investigated the role of autophagy that is induced in response to one of the standard cares of therapy, Fulvestrant plus Palbociclib. We have shown that autophagy inhibition, either pharmacologically or genetically, leads to a modest sanitization after the treatment with Fulvestrant plus Palbociclib, suggesting that the role of autophagy is largely non-protective and that autophagy targeting is not an effective strategy. In parallel with autophagy induction, we observed that Fulvestrant plus Palbociclib drives the cells into a state of growth arrest, which with ß-galactosidase staining, C12 –FDG quantification as well as PCR, have shown that it is a state of senescence from which the cells escape between day 12 and 18. Therefore, our main goal was to either eliminate or at least extend suppression of senescent tumor cell populations.

Initially, we tested a number of conventional senolytics, trying to eliminate the senescent population that is induced by Fulvestrant plus Palbociclib, including the drugs that target BCL2 family protein as ABT-263 as well as ABT-199; however, these strategies did not affect the viability of the FP-treated population.

Recently, much attention has been directed toward epigenetic dysregulation and its possible connection with senescence. One of the main strategies that has recently attracted attention in preclinical studies associated with potential senolytic activity is the targeting the BET family proteins, including BRD4, which have been proven, together with its downstream effector c-Myc, to be upregulated in breast cancer [154, 155, 157, 158, 164].We investigated ARV-825, which showed an extension of the growth arrest mediated by Fulvestrant plus Palbociclib with a potential senolytic activity. We confirmed that the ARV-825 effect is not p53-

dependent as shown in T47D cells; furthermore, we confirmed the results in MCF-7 as well as T47D organoid models.

Another BET inhibitor being tested in many clinical trials is ABBV-744. ABBV-744 showed promising results in AML as well as prostate cancer. ABBV-744 extended the growth inhibition mediated by Fulvestrant plus Palbociclib in both a BRD4 as well as p53-dependent manner. Furthermore, ABBV-744 did not increase the extent of senescence that is initiated by Fulvestrant plus Palbociclib, highlighting a special form of growth inhibition mediated by ABBV-744 in which p53 plays a major role. Investigating the relation between BRD4, c-Myc and p53 is one of the principal future directions.

ARV-825 alone also showed non- senescence growth inhibition, which appears to be associated with both BRD4 and c-Myc suppression. One of our future efforts will be to investigate the nature of this growth arrest. Furthermore, we plan to investigate the ability of ARV-825 to sensitize Palbociclib and Fulvestrant resistant cell lines as well as the *in vivo* effect of ARV-825 + F+P in different mice models.

Another endocrine therapy that is utilized for ER+ breast cancer is Tamoxifen, which attracted the attention recently in the literature to increase its effectiveness by combing it with BET inhibitors [99, 170]. Tamoxifen in combination with ARV-825 or ABBV-744 shown a drastic increase in the extent of autophagy which it was not associated with senescence. The most promising results have been reported with ARV-825, which showed a significant suppression of the proliferative ability Tamoxifen-treated cells. Therefore, one main future goal is to investigate the effect of ARV-825 in TAM- resistant cell lines, together with understanding the nature of autophagy induced.

Our preliminary results have shown that estrogen deprivation, which is equivalent to the current standard care of therapy, aromatase inhibitors, in combination with Palbociclib drives the MCF-7 cells into state of growth arrest (**Figure 5.1**). Furthermore, ARV-825 addition to the cells subjected to estrogen derivation plus Palbociclib caused a reduction in MCF-7 cells number. Our primary future direction is to investigate; the nature of this growth arrest, the possibility of autophagy induction, the nature of the reduction in cells number, which was also observed with ARV-825 when added to TAM-treated cells, as well as if this reduction could be mirrored in mice models. Mechanistically, we showed that ARV-825 downregulated the expression of BRD2, BRD3, BRD4 as well as c-Myc in different cell lines. One important future concern is to uncover the complete mechanism of action by which ARV-825 induces its action and one possible approach is by using BRD4 and/or c-Myc overexpressing cells.

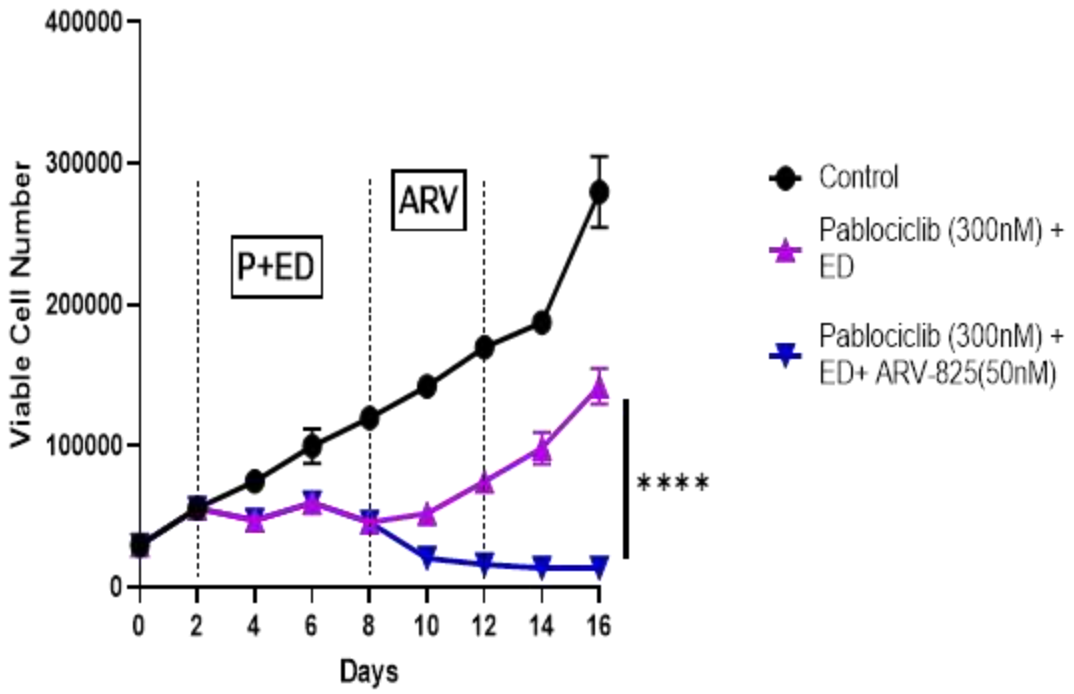


Figure 5.1. Preliminary results in MCF-7 cells. MCF-7 cells subjected to estrogen deprivation (ED) plus Palbociclib (300nM) starting from day 2 to day 8, then ARV-825 was added for 4 days. Cells viability was assessed using tryptan blue over a period of 10 days (**A**). ****P ≤ 0.001 indicate statistical significance of each condition compared to ED plus Palbociclib as determined using two-way ANOVA with Sidak's *post hoc* test.

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